# "INVESTIGATIONS ON WOOD STARCHES."

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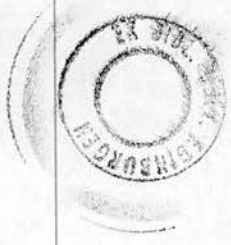

# CONTENTS.

## A C K N O W L E D G M E N T S.

I wish to express my sincere appreciation of the advice and guidance given by Professor E.L. Hirst, F.R.S., under whose direction the following investigations were carried out.

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# CHAPTER I.

## THE GENERAL CHEMISTRY OF STARCH.

### Part I. Historical Introduction.

Over a period of many years a vast number of publications have accumulated dealing with chemical, botanical and technological aspects of starch. The interest thus displayed in this substance is due mainly to its biological significance as the chief reserve carbohydrate of plants as well as to its widespread importance as a foodstuff and its variety of applications to industry and in the home.

The term "starch" refers to polysaccharides occurring in plants in the form of granules, the shape and size of which are characteristic of their botanical source and which give quantitative yields of glucose upon complete acid hydrolysis and mainly maltose upon partial enzymic hydrolysis. The fact that starch yields glucose upon acid hydrolysis was first recorded almost a century and a half ago by de Saussure (1) and since that time no other monosaccharide residue has been proven present as a structural unit within the starch molecule. The first workers to demonstrate the enzymic hydrolysis of starch to maltose were Dubrunfaut (3) who suggested that this sugar was identical with that produced in the initial stages of sulphuric acid

hydrolysis of starch (2) and O'Sullivan (4) who attacked the problem of diastatic conversion of starch to maltose on a quantitative basis.

For many years the significance of these early results with respect to the structure of the starch macromolecule was not recognised until the structures, first of glucose (5,6,7) and then of maltose, had been established. The detailed structure of maltose was elucidated primarily by the identification of 2:3:4:6 tetramethyl glucose and 2:3:6 trimethyl glucose produced simultaneously on hydrolysis of methylated maltose (8,9), but although the methylation technique was first applied to the problem in 1905 by Purdie and Irvine (10), the constitution of maltose remained obscure for some twenty further years. The matter was finally settled by Haworth and Peat (11) when they identified the products of hydrolysis of methyl octamethylmaltobionate as 2:3:5:6 tetramethyl gluconic acid along with 2:3:4:6 tetramethyl glucopyranose, thereby fixing the maltose molecule as D-glucopyranose-4-α-D-glucopyranoside. That the union between the glucose residues is of the type of α-methylglucoside is indicated by the fact that the biose is completely hydrolysed by maltase, an enzyme which is capable of hydrolysing α- but not β-glucosides. Confirmation of the stereochemical nature of the biose junction was furnished by comparison of the magnitude

and sense of the specific rotation of methyloctamethyl-maltobionate (viz. $[\alpha]_D$ + 121°) with that of α-methyl-glucoside (viz. $[\alpha]_D$ + 158°). The fact that hydrolytic cleavage of each of these glucosides causes the specific rotations to decline to lower values (due to the newly-liberated α-sugar falling to an equilibrium value with the β-sugar) enables a stereochemical analogy to be drawn between the configurations of α-methylglucoside and methyloctamethylmaltobionate. The α-configuration of the bionic junction between the gluconic acid residue and the remaining hexose in this bionic acid being thus inferred, the α-configuration of the biose junction in the parent disaccharide, maltose, may also be assumed (12,13).

Since maltose can be obtained as the main product of amylolytic hydrolysis of starch, it seemed reasonable to assume that the starch molecule is constituted as an extended chain of glucopyranose units mutually linked, line ahead, by α-1:4 glucosidic bonds (13), thus -

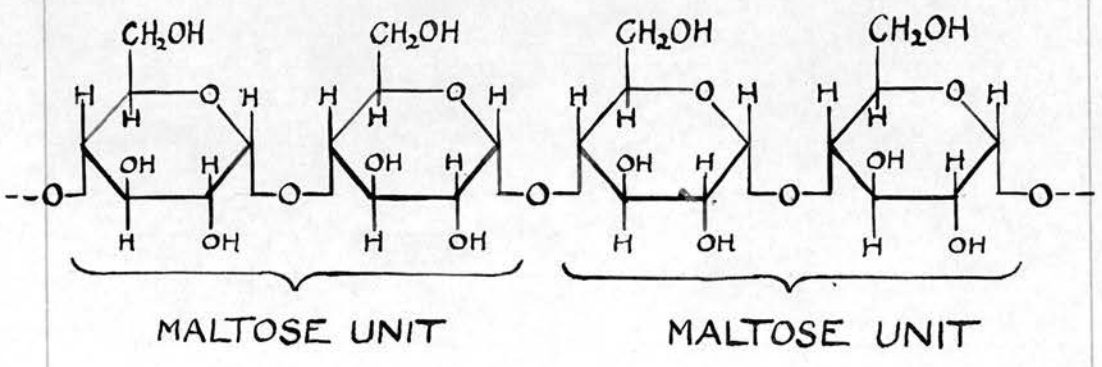
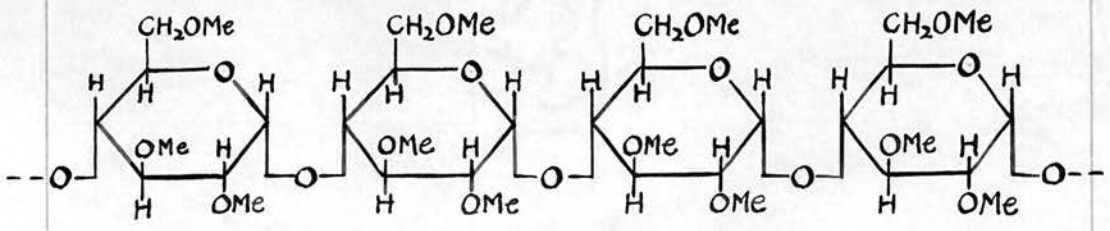



FIG. I.

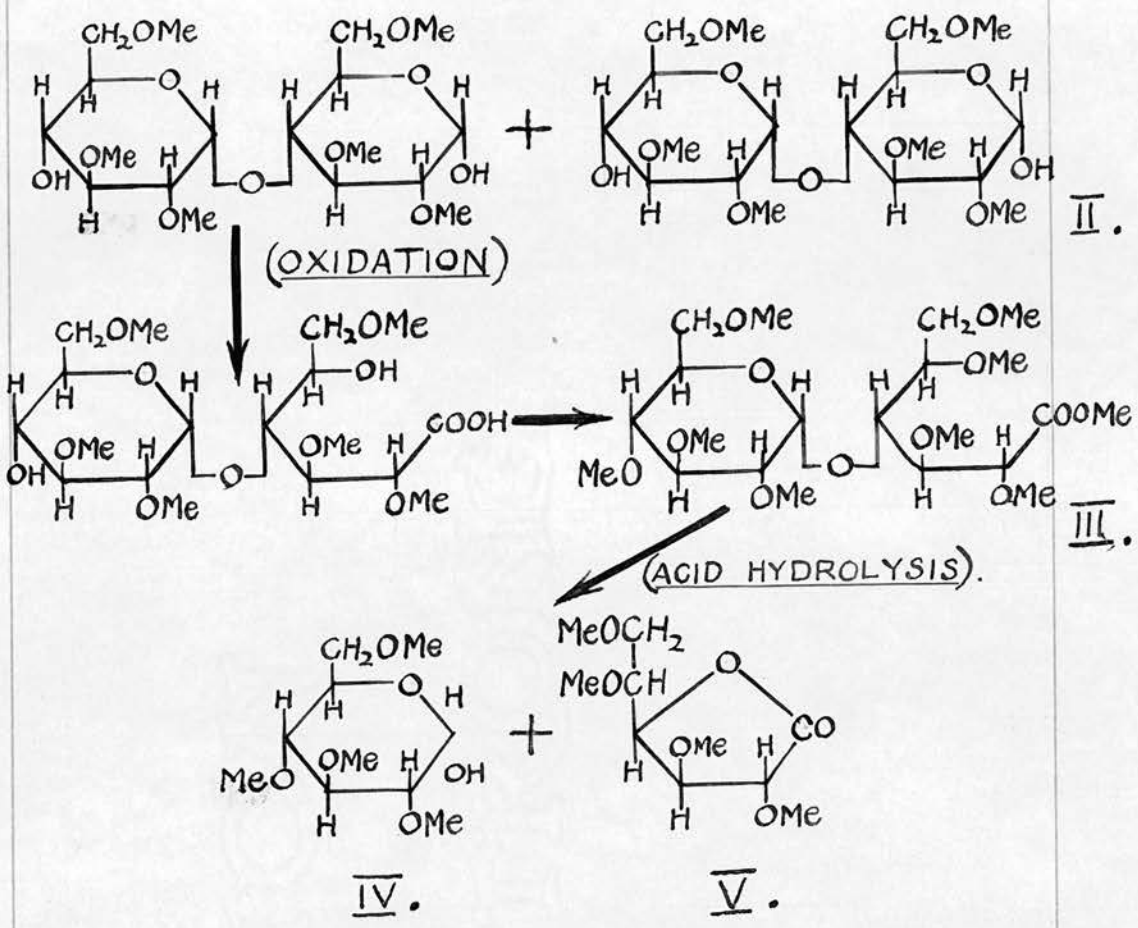
But one of the chief difficulties which delayed the general acceptance of this view was the lack of conclusive evidence that maltose residues exist preformed in starch. Scepticism arose upon the suggestion that enzymes may be present during the degradation of starch which were capable of actually uniting two glucose molecules with the formation of maltose which would then be identified as a mere reversion product of the action of enzymes on starch (14,15). However, Karrer and Nägeli (16) provided purely chemical evidence that maltose units may be obtained direct from starch. This was demonstrated by treating starch with acetyl bromide to yield hepta-acetyl maltosido-1-bromide, a maltose derivative which under identical experimental conditions can be obtained from maltose itself in approximately similar yields.

The above hypothesis that starch is composed of conjugated maltose residues was given experimental proof by Haworth and Percival (17) in 1931 by examination of the products of acetolysis of methylated starch.




Ia. TRIMETHYL STARCH.

ACETYL BROMIDE (AT 16°)




II. III. IV. V.

Hexamethyl maltose (II) was isolated from the acetolysis products and this was hypobromite oxidized, then methylated and esterified to yield methyl octamethylmaltobionate (III) (11), hydrolysis of which gave 2:3:4:6 tetramethyl glucose (IV) and 2:3:5:6 tetramethyl-a-gluconolactone (V), the identification of these latter two products indicating the 1:4 glycosidic nature of the bond between the glucose residues in the hexamethyl maltose (II). The positions of the methoxyl groups were fixed in this compound by the fact that it yielded only 2:3:6 trimethyl glucose on hydrolysis. Thus the presence

in starch of successive glucose residues in the pyranose form joined by α 1:4 linkages was established.

Further evidence, not only of the presence of α-linkages, but of their continuity within the starch chain was provided by Freudenberg's studies on optical superposition in polysaccharides (18). According to Freudenberg, if the starch chain is entirely uniform, its molecular rotation should be an additive property due to the repetition of a constant value contributed by each of its individual constituent glucose units. The following equation was deduced on this assumption:-

$$\frac{[M]n}{n} = [M]_2 - \frac{[M]\alpha}{\alpha} + \frac{2n-1}{n}\left(\frac{[M]\alpha}{\alpha} - \frac{[M]_2}{2}\right) \text{ where } n \geqslant 2 \quad ..(1)$$

where, in the case of methylated derivatives,

$[M]_n$ = molecular rotation of "trimethyl" starch,

$[M]_2$ = " " " heptamethyl methyl,

maltoside = $[\alpha]_D$ (90·2° in chloroform) X M.Wt.(454) = 410.

n = degree of polymerisation of the chain

and $\frac{[M]\alpha}{\alpha}$ is the additive function and represents the rotation-contribution of the repeating-unit within the starch molecule. This value may be calculated by substituting appropriately in the above equation. Thus using the value obtained by Irvine and Macdonald (19) for the specific rotation of exhaustively methylated potato and rice starches (viz. $[\alpha]_D$ + 216° in chloroform),

$$\frac{[M]_n}{n} = \frac{216 \times n \times 204}{n} = +441,$$

and since, for starch, "n" in equation (1) can be considered to be infinitely great,

$$\frac{[M]_\infty}{\infty} = \frac{[M]_n}{n} = +441$$

Substituting for the values of $\frac{[M]_\infty}{\infty}$ and $[M]_2$ in equation (1), the molecular rotations of the following triose and tetrose can be calculated:-

(a) HendeKamethyl maltotriose -

$\frac{[M]_3}{3} = +284$, thus giving a calculated value for the molecular rotation, $[M]_3$, of this compound of +852 as compared with the actual value of +855·4.

(b) TetradeKamethyl maltotetrose -

$\frac{[M]_4}{4} = +323$, thus giving a calculated value for the molecular rotation, $[M]_4$, of this compound of +1292 as compared with the actual value of +1260.

Thus Freudenberg was able, on the one hand, to calculate the molecular rotations of these two methylated oligosaccharides, and conversely, the rotations of the latter made it possible to calculate that of "trimethyl starch." The agreements were close enough, according to Freudenberg, to conclude that, at the most, only one bond in every 30 (and

possibly up to 50 or 60) in the starch chain could deviate from the uniformity (viz. α-glucosidic bonds only) assumed in the deduction of equation (1).

The methylation technique was first applied to the problem of starch constitution in 1920 (20,21), but while the detailed structure of maltose remained obscure, the interpretation of the results of these early experiments was mainly speculative. Irvine and Macdonald (19) isolated a crystalline sugar as the main product of hydrolysis of methylated starch and correctly identified it as 2:3:6 trimethyl glucose, but although this product is consistent with the formulae of starch and methylated starch as depicted in Figs.I and Ia above, such a result in itself fails to discriminate between glucopyranose and glucofuranose residues as the fundamental building units of the starch molecule. The issue was decided by the acetolysis experiment described above.

Methylation of starch by Irvine and Macdonald (19) was accomplished by the repeated application of dimethyl sulphate to the original starch dispersed in alkaline medium, but as the process was found to be experimentally difficult, a modification of the technique was introduced by Haworth, Hirst and Webb (22) in which advantage was taken of the solubility of "triacetyl starch" in acetone. Potato starch was first acetylated by means of a mixture of acetic acid

and acetic anhydride in the presence of thionyl chloride as catalyst. A solution of the product in acetone was then treated with dimethyl sulphate and alkali, when simultaneous deacetylation and etherification proceeded smoothly and rapidly. In this way, 36% of methoxyl was introduced in one operation and a progressive increase in the methoxyl content was observed on each subsequent methylation, six separate treatments in all being required to raise the methoxyl content to 44%. Methanolysis of the final product followed by hydrolysis of the methyl glycosides provided confirmation of the earlier results, in that crystalline 2:3:6 trimethyl glucose was again isolated as the main yield.

The accumulated evidence outlined in the foregoing pages thus provided proof of the presence in the starch molecule of chains of α-glucopyranose residues mutually linked as in maltose. Evaluation of the average lengths of these chains in the case of potato starch was accomplished in 1932 by Hirst, Plant and Wilkinson (23) by application of the gravimetric method of "end-group" assay which was developed by Haworth and used in the first instance for cellulose (28). Acetylated potato starch was simultaneously deacetylated and methylated as described above, and the product hydrolysed to a mixture of methylated glucoses from which tetramethylglucopyranose

was quantitatively separated by a process of solvent extraction followed by fractional distillation in high vacuum of the corresponding methyl glycoside. The yield calculated as tetramethyl glucose amounted to 4-5% of the total weight of the syrupy mixture of methylated sugars obtained as the hydrolysis product. Since only those glucose units occurring at the non-reducing end of a starch chain can give rise to tetramethyl glucose under the above conditions, the average chain length of the potato starch molecule was calculated as 24 anhydroglucose units, and the formulation of methylated starch was given in the following expression:-

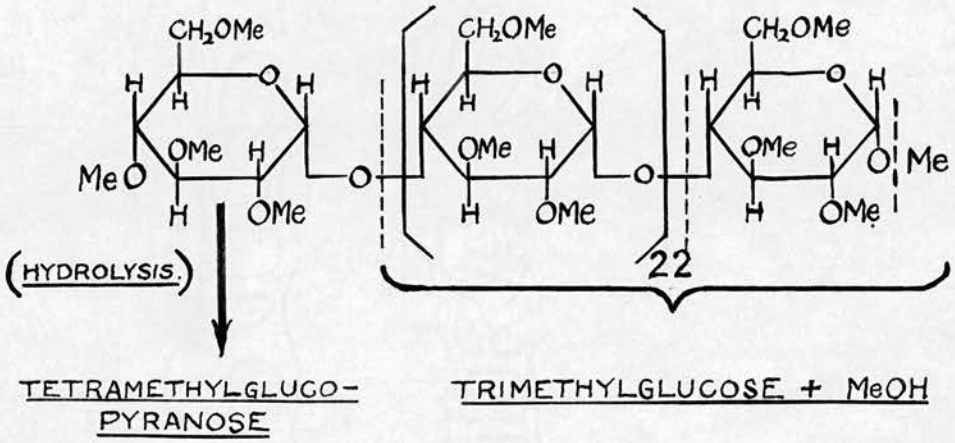



FIG. VI.

It was pointed out, however, that the postulation of such a relatively small molecule for unsubstituted starch was not compatible with certain other observed facts pertaining to the reducing end of the starch chain. The presence in starch of 4-5% of reducing

end-groups should be measurably manifest by the use of appropriate reagents, but it had long been known that starch was non-reducing towards Fehling's solution and showed negligible iodine uptake on hypoiodite oxidation. These facts, of course, suggested a very much greater chain-length than was indicated by the methylation technique of end-group assay. An attempt to reconcile the conflicting data was made at this time by suggesting that the reactivity of the reducing end-groups was masked by the formation of micelles or aggregates of starch molecules by interlocking of the individual chains. This idea of physical aggregation of discrete chemical units (29) to form supermolecular assemblages of great particle size gained favour in the succeeding 2-3 years as it appeared to explain certain observations and anomalies such as the results of physical methods of mol. wgt. determination which are discussed below.

The results of many subsequent experiments indicated the presence of 4-5% of tetramethylglucose amongst the hydrolysis products of methylated whole starches from a wide variety of sources using both the original method outlined above and an improvement in the technique later elaborated by Hirst and Young (30). The chain-lengths thus calculated for the various starches are reviewed in Table I.

| Source of Starch. | Number of Glucose Residues per one Non-reducing Terminal Residue. |
|---|---|
| Potato (23) | 25 |
| Maize (24,25) | 25 |
| Waxy Maize (49) | 26 |
| Canna (47) | 27 |
| Rice (35) | 30 |
| Horse chestnut (34) | 28 |
| Wheat (34) | 24 |
| Banana (26) | 24 |
| Acorn (27) | 30 |

TABLE I.

The fact that a more or less common end-group assay was obtained for all starches examined was of great importance because not only did the value imply that chains of glucose residues of finite length occur in starch, but that the average length of these chains is a characteristic function of starch whatever its botanical source.

The application of physical and physico-chemical methods to the determination of the molecular weight of starch indicated a molecule of much greater magnitude than would be supplied by a simple chain of 24-30 anhydroglucose units having a mol. wgt. of the order $5 \times 10^3$. For example, sedimentation equilibria for dilute potato starch solutions as determined in the ultracentrifuge according to the method of

Svedberg corresponded with mol.wgts. over the range $1 \times 10^5$ to $4 \times 10^6$, varying according to the mode of dispersion and pretreatment of the starch (31,32, 33). Again, mol.wgts. of starch derivatives of half to three quarters of a million (34,35) were calculated from viscometric data by the use of an equation evolved empirically by Staudinger (36,37) for linear polymeric series, namely,

$$\eta_{sp.} = K_m M c,$$

where $\eta_{sp.}$ = specific viscosity,

M = mol.wgt.

c = conc. in mols of basic structural unit of the polymeride per litre,

and $K_m$ = proportionality constant which shows fair constancy for a given polymeric series but varies for different series, solvents and temperatures. Concentration effects can also cause serious fluctuation of this constant. For these reasons, various modifications of the original equation are now in use which cater for the variables (38,39,40,35). Confirmation of the high order of magnitude of the starch molecule was provided by the results of osmotic pressure measurements which were also used as a basis for the evaluation of the constant, $K_m$, in Staudinger's viscosity equation (37,41). Dilute solutions of starch (42) and of starch derivatives (37,41,43) gave comparable mol.wgts. by this method of the general

order of some hundreds of thousands.

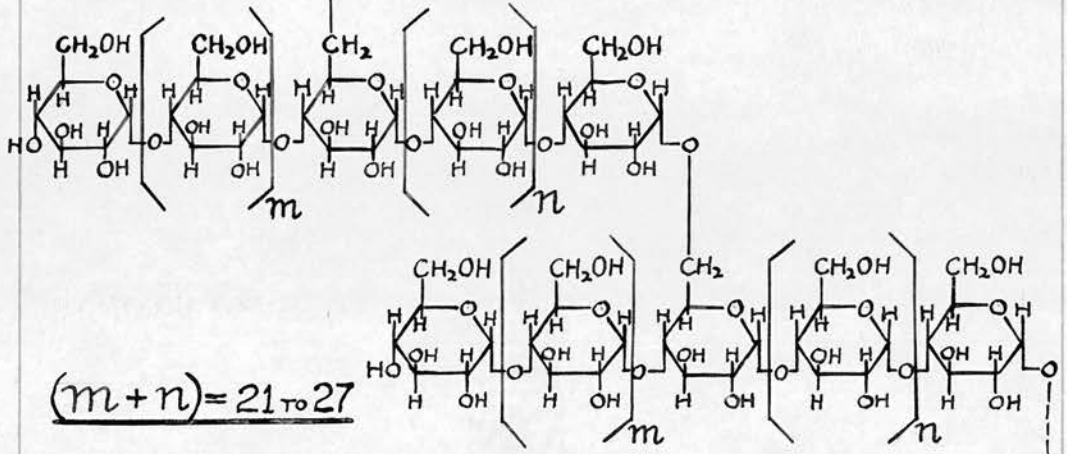
Richardson advanced an hypothesis for the correlation of the results of these physical mol.wgt. determinations of starch with the results of a purely chemical technique (44) which he and his co-workers developed for the estimation of the reducing end-group of the molecule. He suggested that the negligible reducing powers which had previously been recorded for starch by the use of alkaline copper reagents were due to the slight solubility of $Cu_2O$ in the alkaline medium and that below a certain "threshold" quantity of starch, no $Cu_2O$ was precipitated. Saturation of the solution with $Cu_2O$ was therefore ensured during the estimation by the addition of a known quantity of glucose, the excess of $Cu_2O$ precipitated being allowed for by subtracting the weight of this product found in a separate blank experiment with glucose alone. On the basis of the values obtained and assuming one reducing end-group per molecule, Richardson proposed that starch consisted of unbranched chains of varying lengths, the average degree of polymerisation of these chains lying between 500 and 1500 glucose units. He further suggested that the shorter chain lengths of 24-30 glucose units as determined by the methylation technique were due to degradation of longer chains during acetylation followed by methylation.

However, subsequent experiments conducted by Haworth, Hirst and co-workers (34,35,45,46) and by Hassid and Dore (47) proved that irrespective of the mode of preparation of the methyl derivative, i.e. whether it was prepared via the acetate or directly from granular starch or whether methylation was performed in an atmosphere of air or of nitrogen, and regardless of its viscosity in solution, a proportion of tetramethyl glucose corresponding with a chain length of 24-30 glucose units was always obtained on hydrolysis. These observations precluded the possibility of extensive random hydrolysis of long chains during alkylation and served to emphasise the fact that comparatively short chains of glucose residues are of fundamental significance to the architecture of the starch molecule. Furthermore, Richardson's criticism of the methylation technique was not consistent with the fact that hydrolysis of methylated starches for which viscosity and osmotic pressure data suggested extremely high mol.wgts. still provided 4-5% yields of tetramethyl glucose (34,35). Other experiments by Richardson and Higginbotham (48) showed, however, that at least some modification of the starch macromolecule could be incurred by the method employed earlier by Haworth's school for acetylation prior to methylation ( and which was briefly outlined on pp.8,9 of this thesis). This fact

is further exemplified by comparison of the specific viscosities recorded for methyl derivatives which were methylated via the acetate (49) and those which were obtained by direct application of the methylating reagents to the original granules (34,35), the former procedure yielding products of appreciably lower specific viscosity. For this reason primarily, the practice of acetylating polysaccharides to facilitate subsequent methylation was abandoned in later investigations where the use of undegraded derivatives was essential for finer constitutional studies.

In 1937 Haworth, Hirst and Isherwood (50) advanced a common type-formula for starch and glycogen which accommodated most of the known experimental facts relating to these polysaccharides and which gave expression to the fact that the mol. wgts. of starch and glycogen as determined by physical means are many times greater than the chemically established unit-chains. A similar structure had been postulated 3 years earlier by Haworth and Hirst (51) for xylan and was referred to for convenience as the "laminated" formulation; it is represented thus for starch:-

GLYCOSIDICALLY ATTACHED TO ANOTHER REPEATING-UNIT



$(m+n) = 21$ TO $27$

GLYCOSIDICALLY ATTACHED TO ANOTHER REPEATING-UNIT.

HAWORTH-HIRST "LAMINATED" FORMULA FOR STARCH

FIG. VII.

and may be illustrated schematically in the following diagram:-

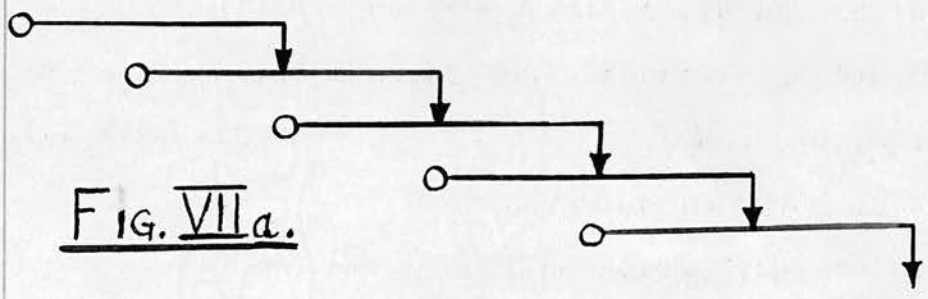


FIG. VIIa.

where the straight lines represent chains of 24-30 glucose residues, the arrows represent lateral glycosidic linkages between the chains, and the circles, non-reducing end-groups. Such a structure possesses only one reducing end-group (R) per macromolecule and obviously estimations of chain-length based on determination of the reducing power of the molecule

would bear no significant relationship to the chain-length of the relatively short repeating-units. Thus the suggestion that the potential reducing end-groups of the unit-chains are involved in glycosidic union with an hydroxyl group of a non-terminal glucose residue in an adjacent repeating-unit reconciled the negligible reducing properties of starches and the results of Richardson's experiments with the results of the Haworth end-group assay.

Strong evidence that the repeating-units are, in fact, bound together in the starch molecule by normal covalent bonds such as occur in, for example, the disaccharides, as opposed to association of the unit-chains by hydrogen bond formation, was provided by an investigation of the kinetics of "disaggregation" of methylated starches (35,52,26). The term "disaggregation" as applied in starch chemistry refers to the preferential breaking, under suitable conditions, of the bonds which unite the repeating units (known as inter-unit bonds) without affecting the glycosidic links between adjacent glucose members within the repeating units themselves, or in other words, it refers to the separation intact of repeating units from large aggregates of these units. This effect was achieved by Hirst and Young by treating methylated starches with aqueous methanol containing oxalic acid (pH 1·8) at temperatures of 60°-70°.

In this way, methylated rice starch of mol.wgt. $5 \times 10^5$ was ultimately disaggregated to molecules of much smaller mol.wgt. (ca. 20,000) which were not further attacked by the oxalic acid and in which the proportion of non-reducing end-groups remained unchanged. Further evidence that the glycosidic linkages within the unit-chains were not broken during the process was provided by the observation that the specific rotation of the products remained constant throughout, whereas the formation of starch dextrins involving cleavage of 1:4 linkages was known to be accompanied by a decrease in specific rotation. The fact that there was a definite increase in reducing power without hydrolysis of α-1:4 linkages within the repeating-units provided firm grounds for the suggestion that the inter-unit bond does involve a glycosidic group. The reaction velocity and activation energy of the disaggregation process were found to be of the same order as those calculated for the hydrolysis of normal glycosidic links and thus the covalent nature of the inter-unit bonds was inferred.

| e.g. | $k_{60°}$ (sec.$^{-1}$) | E(Cal.). |
|---|---|---|
| Disaggregation of Methylated Chestnut starch | $3{\cdot}5 \times 10^{-6}$ | 20,700 |
| Hydrolysis of Inulin | $15{\cdot}6 \times 10^{-6}$ | 25,500 |
| " " α tetramethyl methylglucopyranoside | $4{\cdot}09 \times 10^{-6}$ | 19,840. |

TABLE II.

On the other hand, the activation energy for the rupture of the hydrogen bond, -O-H----O- , has been calculated as 5000-8000 Cal. The possibility was not overlooked, however, that a large number of hydroxyl groups in the repeating-unit of free starch may form hydrogen bonds with corresponding groups of another repeating unit and thus provide very stable union between the unit-chains. But it was pointed out against this view that the hydroxyl groups of starch can be substituted by methoxyl groups, and since there is little change in mol.wgt., this process must take place without the disruption of the molecule which would be expected if free hydroxyl groups facilitated bonding in the original molecule. Again, it was suggested that some hydroxyl groups may escape methylation by the methods used, and it was proposed that these free hydroxyls may bind the repeating units together through hydrogen bonds in the methylated products. However, the results of other experiments (e.g. of Freudenberg and Boppel (53) and of Hassid and McCready (54)) showed that only 4-5% of dimethyl glucose was obtained from exhaustively methylated starches and this proportion, together with a free hydroxyl at the reducing end-group, would correspond with only two free hydroxyls per repeating-unit. Comparative experimental evidence indicated that if two such

hydroxyl groups formed hydrogen bonds, such pairs of bonds should be readily disrupted in water by a reversible process and that equilibrium should be rapidly established. No such reversibility or dissociation, however, was observed with methylated starches.

From the foregoing data, then, it was evident that hydrogen bonding through hydroxyl groups is not primarily involved in the formation of the starch macromolecule, and that the inter-unit bonds broken during disaggregation are primary valencies or, more specifically, glycosidic links. In this connection, it is interesting to recall that from their osmometric data, Carter and Record (43) observed that changes in degree of aggregation with varying concentrations did not occur, and inferred from this that the forces responsible for the particle sizes of polysaccharide derivatives in general should be of a more permanent nature than those normally encountered in associated organic substances.

One of the first clues which led to the postulation of the laminated formula for starch and glycogen was the isolation of a dimethyl glucose from the products of hydrolysis of methylated starches and glycogens (50). The presence of this sugar had been detected in earlier experiments (23) but it was thought to have had its origin either in undermethylation

of the polysaccharide or by partial demethylation of 2:3:6 trimethyl glucose by the hydrochloric acid used for the hydrolysis of the methylated polysaccharides. The former suggestion, in particular, seemed a very likely explanation of the source of the dimethyl sugar since the difficulty of complete methylation of starch was fully recognised. For this reason, the presence of this fraction amongst the hydrolysis products was interpreted with considerable caution. Its structural significance was ultimately confirmed when it was isolated, and identified as 2:3 dimethyl glucose, from amongst the products of hydrolysis of (a) exhaustively methylated starches having methoxyl contents almost equal to the theoretical value of 45·6% for $C_6H_7O_2(OCH_3)_3$ (53,54,55) and (b) disaggregated methylated starches (56) which were remethylated to substitute any hydroxyl groups which had become exposed during disaggregation; methylation was found to proceed readily to the full extent of the theoretical value. The fact that 2:3 dimethyl glucose was isolated in each case makes it obligatory for the lateral glycosidic links between the repeating-units to engage carbon atom 6 of non-terminal glucose residues in successive adjacent chains. Yields of 4-5% and of 3% of the dimethyl sugar obtained respectively from fully methylated starches and from remethylated disaggregated methylated

starches are consistent with the view that one such linkage (viz. 1:6') occurs per repeating unit.

Myrbäck and Ahlborg (57) also contributed evidence of a 1:6 glycosidic linkage by demonstrating the presence of isomaltose (D-glucose-6-α-D-glucoside) amongst the products of hydrolysis of starch by means of acid solutions under such conditions that no reversion was alleged to be possible. Recently, confirmation of this result was provided by American workers (58) who isolated isomaltose as its crystalline octa-acetate by controlled acid hydrolysis of a starch fraction (amylopectin). Controlled enzymic hydrolysis of starch has also been shown to yield isomaltose (57), the identity of which has been established by preparation of its crystalline derivatives, and it has been proven with reasonable certainty, by means of appropriate control experiments, that this product was not merely adventitious (59). Again, by the action of enzymes (takadiastase and malt-amylases) upon whole corn starch, a syrupy tri-saccharide fraction was isolated and evidence recorded for the presence of an α-1:6 linkage in this fraction, but in this case, the structural significance of the yield has not yet definitely been established (60).

Although much further investigation is required in this particular field, the above combined evidence strongly suggests the presence of α-1:6 cross-linkages

in starch, but in the absence of definite quantitative data, the question of whether all or only some of the interunit bonds are represented in this manner has been raised from time to time, other possibilities being union through 1:2' and 1:3' positions. For example, an as yet unconfirmed report has been recorded of the isolation of an α-1:3' linked disaccharide from the products of enzymic hydrolysis of starch (61). Again, hydrolysis of various periodate-oxidised starches and subsequent estimation of unchanged glucose liberated provides evidence which suggests that some 20% of the inter-unit bonds may be other than 1:6' linkages. The interpretation and scope of this technique will be discussed more fully in the next section.

Certainly the results of the disaggregation experiments suggested the existence of at least two types of inter-unit bonds, one of which is unstable to the action of aqueous methanolic oxalic acid and which is thus ruptured to yield molecules of mol.wgt. ca. 20,000 which contain several repeating units which are, in turn, bound together by inter-unit bonds which are resistant to further attack by the oxalic acid, namely, the α-1:6 glycosidic linkages referred to above. It is significant that attempts to further disaggregate this fraction (mol.wgt. 20,000) resulted in breakdown within the repeating-units themselves,

thus suggesting that the α-1:6 linkages in the disaggregation product are approximately as stable as the α-1:4 linkages. Indeed, there is considerable evidence available which indicates that the α-1:6 linkage possesses a somewhat higher degree of stability towards acids than the α-1:4 type. This conclusion could, for example, be inferred by comparison of the activation energies recorded by Moelwyn-Hughes (62) for maltose and cellobiose with that for gentiobiose. Again, reports on the constitution of the polysaccharide dextran (which consists of glucose units mutually linked by α-1:6 bonds) referred to the considerable resistance which the methyl derivative of this polysaccharide displayed towards the usual hydrolytic reagents, and it was observed that methylated starch was appreciably less stable toward these reagents (63). This observation recently received confirmation from the results of Swanson and Cori (64) who found that the α-1:6 linkages of dextran possess a lower velocity constant for acid hydrolysis than the α-1:4 linkages of the starch components (amylose and amylopectin) under identical conditions. Myrbäck also recorded that the isomaltose type of linkage was hydrolysed at a much slower rate than the maltose linkage (65).

This evidence seems to emphasise the fact that the true repeating-unit of starch is not the simple

unit-chain containing some 24-30 glucose residues, but an aggregate of several such unit-chains inter-linked through α 1:6 bonds. These larger repeating units, in turn, appear to be joined through glycosidic links which are ruptured somewhat more readily than either the 1:6 or 1:4 bonds.

However, the general applicability of the laminated formulation for starch as a working hypothesis was not greatly affected by considerations of the precise mode of glycosidic inter-unit bonding. It further served to explain, and in turn received support from the results of enzymic degradation of starch. Wijsman (66) was able to demonstrate the existence of at least two different amylases in barley malt extract by discovery of the fact that they possessed different rates of diffusion on a starch-gelatin plate. A drop of malt extract was placed on such a plate and after the enzymes had been allowed to act for several days, the plate was "developed" with dilute iodine solution. Most of the surface was thus stained deep blue, but where the drop of malt extract had been placed was a colourless disc surrounded by a reddish-coloured annular ring. The enzyme component which was thus shown to possess the greater diffusion rate and to degrade starch to products giving a reddish coloration with iodine was called β-amylase. The slower-diffusing enzyme was

called α-amylase. It is now generally agreed that at least two main types of starch-splitting enzymes exist and that those occurring in barley malt are fairly typical of amylases from other sources (67). Moreover, species of these two enzymes have recently been isolated in the crystalline state (68,69).

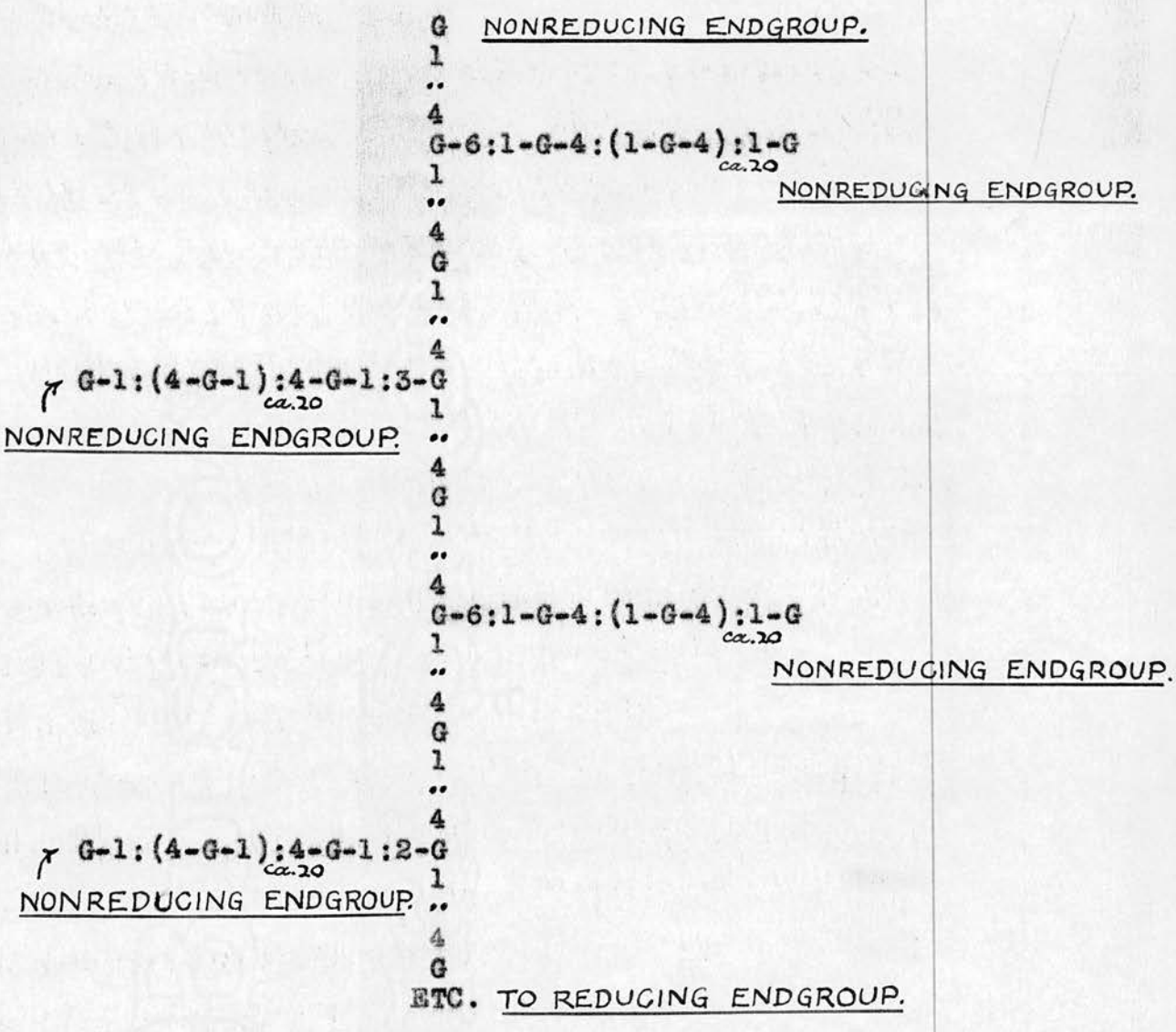
Many workers have confirmed the fact that β-amylase preparations degrade starch to a dextrin (known variously as dextrin-A, α-amylodextrin, erythrogranulose and "limit dextrin") isolated in 40% yields by weight of the original starch, while maltose in approximately 60% yields accounts for the remainder (70-73). Dextrin-A as such, was found to be resistant to further activity by β-amylase. The question of why β-amylolysis should cease when only 60% of a theoretically possible yield of maltose had been liberated provided further grounds for the support of the laminated structure, in the following way. Methylated dextrin-A was shown to have a high mol.wgt. (ca. 79,000 representing a D.P. of 400 glucose residues) and on hydrolysis was shown to yield ca.10% of "end-group" (tetramethyl glucose) corresponding with a unit-chain of 11-12 glucose residues (72,73). These observations indicated that dextrin-A possessed a high degree of aggregation of shortened unit-chains and have been explained on the grounds that β-amylase effects progressive removal of maltose

residues from the non-reducing ends of the unit-chains of the starch molecule (74), and that this activity continues until an impediment is encountered which prevents further attack by the enzyme. These impediments or "blocking-points" are thought to be caused by the polymeric links joining the repeating-units (75,73). Obviously, the glucose units involved in the inter-unit bonding do not conform to a free maltose configuration, being substituted at $C_6$ (or other position) and it seems likely that the enzyme is unable to attach itself at this "anomalous" part of the chain. At this point, then, enzyme action ceases, leaving the remainder of the macro-molecule as dextrin-A.

A preliminary account of this work has been included here to illustrate how the results harmonised with the postulation of the laminated formulation for starch, but will be discussed further in the following section in the light of more recent developments in starch chemistry.

Among other formulae which have been proposed for starch, the so-called "comb" formulation of Staudinger may be mentioned at this stage as also representing most of the known facts fairly well (37, 42). Staudinger assumed the starch molecule to consist of numerous side-chains attached to a

relatively short "principal" chain through 1:6, 1:3 and 1:2 α glycosidic linkages, thus:-




(G = glucose)

FIG.VIII. STAUDINGER'S FORMULA.

As Myrbäck pointed out (76), "if the Staudinger formula is to explain the action of β-amylase, it is necessary to assume that the side-chains are shorter (10-12 units) and are situated at greater intervals

(8-10 units) on the principal chain."

The essential difference between the laminated formula and that of Staudinger is that the former possesses no central or principal chain. It is this feature in the Staudinger structure which renders interpretation of the results of disaggregation experiments difficult. To explain the formation of the product of mol.wgt.20,000 containing several non-reducing end-groups in unchanged proportion, it would be necessary to assume rupture at fairly regular intervals of some of the 1:4 linkages in the principal chain only, while the points of attachment of the side-chains to the main chain, as well as the 1:4 linkages in the side-chains themselves, should remain intact. Such an unqualified assumption is hardly tenable.

Non-carbohydrate Constituents and Impurities Associated with Starch.

Variable but small quantities of phosphorus may be detected in most natural starches, the content usually lying within the range 0·01 to 0·2% P. The phosphorus of cereal starches is not chemically combined with the macromolecule and may be removed by repeated extraction with warm water and alcohol; α and β glycerophosphates have been identified in the

extracts. Schoch has shown that the removal of the phosphorus content of wheat starch, possibly as a phospholipid, is easily accomplished by extraction with hydrophilic fat solvents (77). Other starches, such as those of potato and arrow root, contain esterified phosphorus, a fact which was demonstrated by the identification of glucose-6-phosphate amongst the products of their hydrolysis by both acids and enzymes (79,80,81). Such esterified phosphorus may be removed by heating aqueous solutions of these starches under pressure or by the action of enzymes (e.g. brain phosphatase (82)). Samec (82,83) maintains that the removal of esterified phosphorus affects certain physical properties of the starches concerned, in particular, those of viscosity and paste-formation which are thus reduced. He found that the phosphorus of corn amylopectin (largely in the form of phospholipid impurity) could be removed with little change in the physical properties of the amylopectin. Yet removal of esterified phosphorus from potato amylopectin yielded a product whose physical properties were much more closely comparable with those of corn amylopectin. Conversely, conversion of some of the free hydroxyl groups of corn amylopectin to the phosphate ester was reported to effect increase in viscosity, making the product more comparable in this regard with the amylopectin of

of potato starch.

It appears that phosphorus is involved in the biogenesis of starch within the plant, since Hanes has demonstrated the synthesis in vitro of polysaccharides closely related to starch by the action of potato phosphorylase on glucose-1-phosphate (171).

The presence of ca.0·9% silica in the granules of various starches was pointed out by Ling and Nanji (84) who later suggested that it was organically combined as an ester with the polysaccharide molecules (85).

Fatty acids and other fatty materials are associated particularly with the cereal starches to the extent of some 0·6%. In a series of papers (e.g. 86) Taylor and his co-workers advocated the hypothesis that fatty acids such as palmitic, oleic and linoleic acids were chemically combined as esters with corn, wheat and rice starches. This view was based mainly on the fact that the fatty acids were not directly extractable on prolonged treatment with such fat solvents as ether and carbon tetrachloride and were recovered only after hydrolytic treatment of the starches. Schoch (77) and other workers (78) later demonstrated that the fatty acids were present merely as adsorbed impurities which were distributed throughout the starch granule and loosely bound to the carbohydrate molecules by polar adsorption.

Schoch reasoned that in the choice of a suitable solvent for extraction of fatty acids from starch, two major considerations were involved, firstly, of course, that the solvent must be a tolerably good fat-solvent and secondly, that it must be sufficiently hydrophilic to penetrate the carbohydrate lattice. The solvent molecules were thus preferentially adsorbed on the starch by virtue of their hydrophilic loading and the fatty acids simultaneously discharged and dissolved by excess of the solvent. Effective solvents were found to be, for example, the lower aliphatic alcohols, particularly aqueous methyl alcohol, and also aqueous dioxan. The latter pre-requisite mentioned above was the reason postulated for the failure of such hydrophobic fat solvents as ether and hydrocarbon solvents to effect extraction of the fatty acids. This property of starch to adsorb various polar materials was employed by Schoch for the separation of the components of natural whole starches (87).

In connection with the small proportion of protein impurity ($N \simeq 0{\cdot}05\%$) usually associated with natural starches, Stacey (88) has suggested that it originates from the enzyme system responsible for their biosynthesis. These residual nitrogenous constituents may be removed as flocculent ppts. by boiling with dilute acetic acid and they appear to

resemble denatured phosphorylase preparations.

All starches, after thorough drying, readily reabsorb moisture to the extent of some 10-18% by weight of the actual carbohydrate on exposure to the atmosphere, an equilibrium value ultimately being established. The crystalline structure of granular starches appears to be closely related to their moisture content because removal of water by gentle heating <u>in vacuo</u> over $P_2O_5$ causes the X-ray diffraction pattern to change from a crystalline to an amorphous diagram.

## Part II. Review of Recent Progress in the Chemistry of Starch.

Within the last decade the long-debated question of the heterogeneity of starch has been decided beyond reasonable doubt and the great majority of workers now agree that most native starches consist of two major components to which the names "amylose" and "amylopectin" have been assigned (89). Each component possesses its own highly characteristic physical and chemical properties, and methods have been devised for their separation and their isolation in the pure state from natural whole starches. Amylose has been shown to constitute some 20% of most natural starches and is characterised by its property of giving an intense pure blue coloration with iodine, by its spontaneous precipitation (retrogradation) from aqueous solution, and also by its complete conversion to maltose by the action of β-amylase preparations. Amylopectin, on the other hand, gives a purple or violet colour with iodine, forms comparatively stable pasty gels with cold water and is only partially converted to maltose by β-amylase. This diversity between the properties of amylose and amylopectin is manifested by a relatively simple though distinct difference between the chemical constitutions of the two components. Amylose was reported by K.H. Meyer (90) in 1940 to consist of long

and essentially unbranched chains of some hundreds of glucopyranose residues united through α-1:4 glycosidic linkages, while the term "amylopectin" is assigned to the huge branched structures which were previously postulated for whole starch and which were discussed in that light in Part I of this chapter.

The isolation of each of the two components in more or less pure form facilitated the development of methods of assay of their mixtures either in natural whole starches or during the course of their separation (fractionation) from whole starches. These methods were based on the above-outlined differences in the properties of the two components. For example, Meyer and co-workers (91,92) and later Hassid and McCready (94), used the difference in the extent of β-amylase hydrolysis of the two components as a criterion for the assay of starches and starch fractions. Conversion of amylose to maltose by β-amylase was found to approach 100% (95,96) provided precautions were taken against retrogradation of the amylose to the insoluble condition before the enzyme action was complete (97,76). β-Amylase conversion of amylopectin, however, was found to cease at about 50-55% (98). Hence, knowing the percentage conversion of the starch or starch fraction under investigation, it was a simple matter to calculate its amylose or amylopectin content. As Meyer (99)

observed in this connection, only some 20-30% of the maltose formed during β-amylolysis of most natural starches arises from the completely converted amylose content, the remaining 70-80% being liberated from the partially degraded amylopectin.

Considerably more accurate and convenient techniques are based on the reaction of amylose with iodine. Hassid and McCready (94) described a method of assay based on the quantitative determination of the intensity of the blue coloration imparted to natural starches and other mixtures of amylose and amylopectin upon the addition of iodine under standard conditions. Quantitative determination on the one hand of the brilliant deep blue coloration yielded by dilute solutions of pure amylose under these conditions, and, on the other hand, of the much less intense coloration given by solutions of pure amylopectin thus allowed a simple calculation to be made of the percentage compositions of the mixtures under consideration. Potato starch, for example, was shown to contain about 20% of amylose. A serious limitation on this method is that it cannot be applied for the accurate analysis of starches in general without first determining the colour intensities yielded under the standard conditions by the specific amylose and amylopectin constituting the particular starch under investigation (158). The original method of Hassid

and McCready was slightly modified by Bourne and Peat (100) who introduced the term "blue-value" (B.V.) in this connection. "Blue-value" is defined as the reading on the logarithmic scale of a Spekker photoelectric absorptiometer when the absorption of light by a solution (contained in a 40 mm. cell) of a polysaccharide stained with iodine under the standard conditions prescribed by Hassid and McCready is measured using Ilford gelatin light-filters (No. 608) transmitting light of the order of 6,800 Å. Correction for the absorption of light by the free iodine is made by filling the "control cell" of the Spekker instrument with an iodine solution of the same concentration as that responsible for the development of the colour in the test sample. Higginbotham and Morrison (102) recorded blue-values ranging from 0·06 to 0·16 for pure amylopectins (lower values appear to be given only by degraded amylopectins (101, 129)) and found the B.V. of amylose to vary between 1·26 and 1·48 according to the variety of the parent starch. The blue-values of natural starches lie between these extremes for amylose and amylopectin, and afford a convenient means of expressing the relative proportions of the two components present in a particular starch.

Of a less arbitrary nature is the technique of Bates, French and Rundle (103) who quantitatively

estimated the percentage of amylose in various starches by utilising the marked difference in the ability of the two components to bind iodine in complex formation. It was found on potentiometrically titrating dilute aqueous solutions of amylose in faintly acidic media with very dilute iodine solutions that the activity of the iodine (as measured from the E.M.F. of the cell, Pt$|I_2,KI|$calomel) remained practically constant on successive additions of this reagent until complex formation with the amylose was complete, and only then did the iodine activity begin to increase. This means that amylose is capable of very firm binding of iodine in complex formation. Amylopectin, on the other hand, possesses comparatively negligible affinity for iodine as exemplified by the progressive increase of iodine activity measured potentiometrically from the first and subsequent additions of the dilute iodine solution to aqueous dispersions of this component.

Potentiometric iodine titrations of dispersions of whole starches provide data which, together with the results of a blank experiment, enable the calculation of both "free" iodine concentration and "percentage iodine adsorbed" at each addition of the standard iodine solution. If these two functions are plotted one against the other with "free" iodine as the abscissa, a graph is obtained consisting of

two essentially straight lines intersecting as shown in Fig.IX.

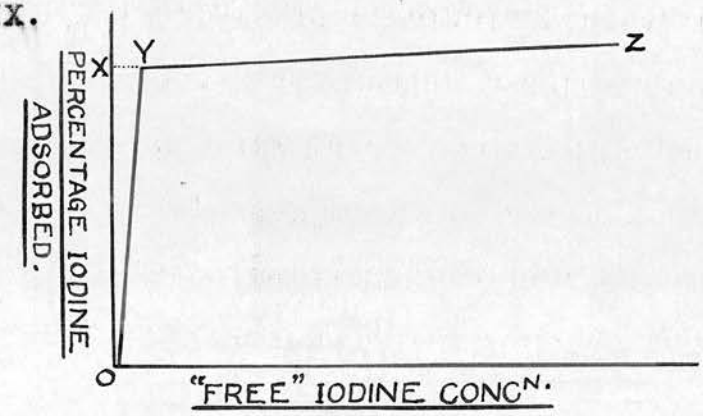



FIG. IX.

The almost vertical straight line 'OY' rising from the origin represents adsorption of iodine by the amylose component while the more gentle slope of the line 'YZ' is probably due to the much weaker association of iodine with amylopectin. Completion of the formation of the amylose-iodine complex is respresented by the point 'Y' to which the value 'X' is the corresponding "percentage iodine adsorbed" exclusively by the amylose content of the starch sample. In passing, it may be mentioned that the very definite break at 'Y' in such curves for whole starches provides strong evidence for the two-component theory of starch constitution, since the abrupt rise in potential which is responsible for the break is characteristic of heterogeneous systems. Higginbotham and Morrison (104) have isolated samples of amylose of exceptional purity from a variety of sources which have been shown by the above technique to adsorb 21·4 - 21·5% of their

own weight of iodine. Hence the percentage of amylose from the above diagrammatic graph for a hypothetical whole starch would be calculated as

$$\frac{100\ X}{21{\cdot}5}$$

The accuracy of this method depends upon the complete absence of impurities which may impair the adsorption of iodine on to the amylose by virtue of their own preferential affinity for that fraction. Thus the fatty acids normally associated with cereal starches have been shown to markedly repress the adsorption of iodine by the amylose component (122, 155). Schoch (105), therefore, recommended exhaustive defatting of such starches by extraction with aqueous methanol or aqueous dioxan prior to analysis. Of course, the absence of other impurities which actually react with iodine or other reagents used in the analysis is also essential. Such an impurity is gallotannin which has been shown in the course of the present work to be strongly adsorbed to the extent of some 4-5% on all wood starches investigated.

By applying their potentiometric technique, Bates, French and Rundle obtained the following percentage values for the amylose contents of various common starches: tapioca 17, rice 17, banana 20·5, corn 21, potato 22, wheat 24, sago 27 and lily bulb

34 per cent. Waxy rice, waxy sorghum, waxy maize and waxy barley starches which were previously known to give reddish or purple colorations with iodine were found to be devoid of amylose, thus confirming the results of Meyer (92,93) in this connection. On the other hand, Peat and co-workers (106) by means of blue-value estimations and β-amylase conversions showed that the starch isolated from the "Steadfast" variety of wrinkled pea consists essentially of amylose, and they confirmed a report by Hilbert and MacMasters (107) that other varieties of wrinkled pea yield starches consisting of some 60-70% of amylose. The starches from varieties of smooth pea were shown to conform more nearly with the majority of seed and root starches so far investigated, in that they contain about 30% of amylose.

The dualistic nature of starch composition had been suspected by a number of earlier workers for a long time prior to the recent conclusive developments. In the absence of effective techniques both of separation and estimation of the components and, therefore, of any decisive evidence, however, the pertinent literature extending over many years presents a confusing record of apparently discordant data from which much conflicting speculation had arisen concerning possible causes for the physical and chemical differences between the starch components.

Probably the first definite suggestion that the starch granule was not chemically homogeneous was made as long ago as 1825 by Raspail (108) who reported the isolation of soluble and insoluble fractions exhibiting different colorations with iodine. Other investigators including Guerin-Varry (1834-36), Nägeli (1826-81), A. Meyer (1881-95) and Gatin-Gruzewska (1908-11) supported this initial observation on various grounds (109, 110), but the most obvious inconsistency which emerges from a review of the earlier communications, as well as those of later date, is the marked divergence in the reported ratios of the two or more components proposed.

The terms "amylose" and "amylopectin" were first introduced early in this century by Maquenne (111) who based his conclusions regarding the heterogeneity of starch upon its behaviour toward the enzyme malt-amylase. He applied the term "amylose" to that component which he observed to be completely decomposed by this enzyme, while the term "amylopectin" was used to designate the other component which Maquenne erroneously assumed to remain unattached. The nomenclature of starch chemistry became progressively more confused due to the indiscriminate use of other terms such as α- and β-amylose, erythro-amylose and amylo-amylose etc. which were employed to designate

alleged fractions of natural starches.

Among the numerous attempts to separate starch into its components the following representative methods may be outlined and discussed according to their merits. A few of the earlier proposed methods met with more or less success while others hold only historical interest. In order to avoid confusion, the terms "amylose" and "amylopectin" are throughout employed uniformly in the light of their present-day significance and substituted, where interpretation is possible, for other terms used in the original literature.

1) Selective Enzymic Degradation. On the basis of his hypothesis regarding the starch components, Maquenne (111) and subsequent workers (84,112) attempted to isolate 'Maquenne's amylopectin' by allowing malt-amylase to completely degrade the amylose component. It was later recognised, however, that there is no constituent of starch which is completely unaffected by amylases, and the final products obtained by Maquenne and others were undoubtedly dextrinised amylopectin fractions.

Other workers, on the other hand, have probably isolated degraded amyloses as flocculent precipitates by treatment of gelatinised cereal starches with certain amylase preparations (113).

2) Selective Retrogradation. Many attempts have been made to effect fractionation from auto-claved starch sols by allowing the unstable amylose fraction to spontaneously precipitate on long standing (110,114). This method, however, has been shown to be unsuitable because the precipitated amylose is invariably contaminated with considerable proportions of amylopectin.

3) Selective Adsorption. Tanret (115) was the first worker to demonstrate that cellulose (in the form of cotton wool) preferentially adsorbs amylose from cold aqueous starch sols. Samec (116) reported that cotton adsorbs some 1·7% of its own weight of amylose. Pacsu and Mullen (117) showed that selective adsorption of amylose occurs also on activated carbon, fuller's earth, and alumina, but reported more effective separations from cold 1% corn starch pastes by using cellulose as the adsorbent. The amylose was recovered from the cellulose by washing with hot water, but again, such samples have been shown to be heavily contaminated with amylopectin (102,105).

Peat and co-workers (118) have recently reported the fact that although aluminium hydroxide is capable of adsorbing whole potato starch almost completely from aqueous solution, it is the amylopectin component, and not amylose, which is thus preferentially adsorbed as an insoluble complex. A technique was elaborated

for obtaining potato amylose in relatively pure form (B.V. 1·35 - 1·40) by precipitation and removal of the insoluble amylopectin-aluminium hydroxide suspension from 3% aqueous dispersions of the whole starch. The yield of amylose was reported to be somewhat variable and always lower than the corresponding yield obtained with the organic precipitants discussed later.

4) Electrophoresis. Samec (119) discovered that fractionation of potato starch could be effected by subjecting an aqueous dispersion of this starch to the action of a weak electric current. The solution slowly lost its turbidity as the amylopectin component (upon which organic phosphate is preferentially esterified (120) and which consequently is negatively charged) underwent electro-migration toward the anode. The potato amylopectin (so-called amylophosphoric acid), thus rendered insoluble, collected as a sediment beneath the anode while the supernatant solution became richer with respect to the phosphate-free amylose fraction. Since few starches only have been shown to contain esterified phosphorus, this method of fractionation is of very limited application. This separation in the case of potato starch appeared to ascribe to phosphoric acid a dominant rôle in the differentiation of amylose and amylopectin, and Samec postulated that esterified

phosphate on portion only of what was otherwise essentially homogeneous carbohydrate material was responsible for the existence of the two components. This idea persisted for many years and was extended to the association of starches with other substances such as fatty acids. For example, Taylor et.al. (121) demonstrated that fractionation of corn starch could be effected electrophoretically in spite of the fact that this variety contains very little combined phosphorus. The separation in this case was credited to esterified fatty acids and it was thought that the components of corn starch differed only in respect of the presence or absence of combined fatty acids. It has now been shown that the fatty acids are merely adsorbed on the carbohydrate lattice (77) and further, that adsorption occurs preferentially on the amylose fraction (122). Thus in contrast to the electrophoresis of potato starch it is the amylose fraction of corn starch which migrates by virtue of its adsorbed anionic impurities.

Separation by means of electrophoresis is slow and incomplete, however, and considerable retrogradation occurs during the process (98). Moreover, the use of this method wrongly assumed that the non-carbohydrate impurities which impart polarity are associated exclusively and uniformly with one component of the starch.

5) Aqueous Leaching of Frozen Pastes. Ling and Nanji (84) claimed to have developed a method of fractionation by first freezing a paste of the starch and then leaching the fibrous mass so formed with successive portions of water at about 60°. Amylose was thus said to pass slowly into solution leaving an almost pure residue of amylopectin.

However, the results of subsequent methylation experiments reported in 1932 by Hirst, Plant and Wilkinson (23) now throw serious doubt on the efficacy of this method of fractionation in that identical yields (viz. 5%) of tetramethylglucopyranose were isolated by methylation and subsequent hydrolysis of each of the so-called starch fractions obtained in this way. The results were interpreted at the time of this investigation on the assumption that Ling and Nanji's method was, in fact, effective in the separation of the two suspected components, and it was concluded that the molecular constitutions of these components were essentially similar. The differences in physical properties were attributed to differing states of hydration and degrees of aggregation of the short chains of 24-30 glucose residues, the term "amylopectin" being applied to what were considered to be the larger colloidal micelles.

Thus a type of deadlock was established in that earlier workers, having no accurate criteria as to

what constituted true fractionation, unwittingly employed methods which were either partially or wholly ineffectual. The inadequacy of many of these methods was, therefore, responsible for the long delay in the recognition of amylose and amylopectin as distinct chemical species. On the other hand, the development of more efficient methods of fractionation was retarded by the lack of sufficient knowledge concerning the individual properties and characteristics of each component in the absence of the other.

6) Aqueous Leaching of Swollen Granules. Starch granules suspended in water at about 60-80° swell greatly without suffering actual rupture, and from the swollen, intact granules crude amylose diffuses out in water-soluble form. The rate of diffusion of the amylopectin molecules is reduced practically to zero by mechanical entanglement due to the branching nature of their structure.

Gatin-Gruzewska (110) first applied this method to potato starch granules using as the leaching agent weakly alkaline aqueous solutions for which Tanret (123) later substituted hot water with some apparent success. Baldwin (124) in 1930 introduced improvements and described two variations designed to yield amylose on the one hand and amylopectin on the other.

The method suffered further slight modification in succeeding years and was applied in 1940 by K.M.Meyer (99) in the following form. The swollen granules (of corn starch) were repeatedly extracted with successive portions of hot water (70°C.) until no further solubles were removed. After separation of suspended amylopectin-containing particles by centrifugation or filtration, the amylose was allowed to retrograde from solution during the course of several weeks at 5°. It was then resolubilised by treatment with dilute sodium hydroxide in the absence of oxygen.

In this way, Meyer was able to isolate samples of amylose and amylopectin which were sufficiently pure for constitutional studies. The samples were methylated and "end-group" assays performed on the products. The proportion of tetramethylglucose obtained from the amylopectin fraction (taken in conjunction with other properties) showed this component to conform with the branched type of structure previously postulated for whole starch (125, 126). On the other hand, the small proportion of "end-group" (ca. 0·3 - 0·4%) isolated from the hydrolysate of the methylated amylose fraction proved conclusively that this component is constituted as long chains containing some hundreds of anhydroglucose units per non-reducing end-group. The mol.wgt. of a

sample of methylated amylose calculated from osmotic pressure data was found to coincide with that calculated on the assumption that only one non-reducing end-group occurred per molecule, thus proving that the majority, at least, of amylose chains in this sample were unbranched. Meyer (127) also found that crude amylose from maize and potato starches could be separated into fractions having mol.wgts. of 10,000 to 60,000 and concluded that amyloses are mixtures of polymeric, homologous series of unbranched chains.

The advantage of hot water leaching of swollen granules lies in the avoidance of high temperatures and the use of chemicals, and therefore little if any degradation of the polysaccharides occurs during the process. However, the method is tedious and Schoch (105) has demonstrated by potentiometric iodine titration of fractions obtained in this way that separation is very imperfect. This perhaps is to be expected since some of the amylose molecules will be so intricately entangled within the mosaic of the granule that their rate of diffusion will be very slow, and so they will appear as an impurity in the amylopectin fraction. On the other hand, contamination of the extracted amylose could occur by a tearing loose of some of the amylopectin molecules composing the surface of the granules. The method is

further criticised on the grounds that retrogradation of amylose to the insoluble condition probably occurs during its extraction.

7) Selective Precipitation with Polar Organic Substances. In 1941 Schoch (87) reported a superior method of starch fractionation of general application based on the adsorptive affinity of amylose for various polar organic molecules containing a hydrophilic grouping such as hydroxyl or carboxyl in combination with a hydrophobic residue. The amylose chains were thought to adsorb such molecules by virtue of their polar attraction for the hydrophilic groupings, the resulting complex becoming insoluble and thus precipitating from aqueous solution due to its hydrophobic loading. n-Butanol was first reported as an effective selective precipitant for amylose, and Schoch accomplished his original fractionations by autoclaving 1-3% starch pastes saturated with n-butanol for 2-3 hours at 18-20 lbs. pressure. Slow cooling to room temperature caused the separation of the insoluble amylose-butanol complex as characteristic micro-spherocrystals which were collected at the supercentrifuge. The non-precipitated amylopectin fraction was recovered from the centrifugate by addition of excess methanol and the resulting precipitate collected by filtration.

More thorough purification of the amylose was

effected by subsequent re-solution of the initial complex in hot butanol-saturated water, slow cooling, and collection of the freshly separated amylose-butanol complex. By repetition of this process contaminating amylopectin could be completely removed.

Other higher aliphatic alcohols such as cyclohexanol (129), n-amyl, n-hexyl and lauryl alcohols also proved of value for fractionation, while "pentasol" which is a commercial mixture of various primary amyl alcohols, was reported to be particularly effective and to provide practically quantitative separation of the amylose component (105, 128).

An important feature to which much of the effectiveness of this method is due is the prevention of retrogradation during the process by virtue of the complex formation of amylose with the precipitating agent. Furthermore, an amylose preparation may be stored as, for example, the butanol complex which in the presence of excess butanol is stable for many months, but on removal of the butanol, the liberated amylose quickly retrogrades to the insoluble form.

An essential pre-requisite to the success of clear-cut fractionations is the complete dispersion of the starch granules in the aqueous-alcoholic medium. To achieve this ideal Schoch recommended autoclaving of the starch pastes and claimed that no

hydrolytic cleavage was thus engendered providing the pH was maintained within carefully controlled limits. Haworth contended, however, that some hydrolytic degradation could, in fact, be caused by the relatively high temperature (120°) of the autoclaving process, and he and his co-workers (101, 129) elaborated a method in which 1-3% aqueous starch pastes (containing 0·1% NaCl) were dispersed by boiling for 0·5 hour with vigorous mechanical stirring. After super-centrifuging, the solutions were cooled to 30° and then saturated with thymol, precipitation of the water-insoluble thymol-amylose complex occurring within the following 2-3 days at 30°. In this way, Haworth claimed to avoid some of the difficulties of the Schoch method and to effect a sharper separation of the starch components. In the case of potato starch, amylose samples of B.V. ca. 1·2 and amylopectin fractions B.V. 0·17 - 0·23 were obtained.

Representatives of many different classes of organic compounds other than alcohols have also been shown to selectively precipitate the amylose component from aqueous dispersions of whole starches. For example, higher fatty acids such as butyric (130), oleic (122) and stearic acids (131) have been employed to some effect, but as mentioned previously, the success of potentiometric iodine titrations for the

subsequent analysis of the fractions obtained, depends upon the absence of traces of fatty acids. The subsequent complete removal of fatty acids from the fractions obtained by means of these compounds has been found so difficult to accomplish that their use is almost precluded for this purpose.

Whistler and Hilbert (130) suggested that the intermolecular attraction between amylose and its selective precipitants may involve hydrogen bonding. Working on this assumption they found that compounds belonging to a variety of classes having electron donor or acceptor or both groups were effective reagents for fractionation of whole starches. Specific examples of such compounds are nitrobenzene, amyl acetate, methyl ethyl ketone, pyridine (132) and certain nitroparaffins such as nitroethane and the nitropropanes.

Continued efforts are being made to achieve the ideal of complete dispersion under the mildest possible conditions prior to actual fractionation with a view to causing less chemical modification of the components and at the same time, to obtaining sharper separations. Higginbotham and Morrison (102) recommended the use of 30% aqueous pyridine as the dispersion medium and reported that almost complete 2-4% dispersions of starches were obtained in this solvent by rapid stirring at 90° for varying lengths

of time according to the variety of starch under investigation. After dispersion, the hot solution was diluted once with hot water to give a 15% aqueous pyridine fractionation medium, an optimum concentration for the separation, on slow cooling, of the amylose-pyridine complex. Pure amylose was obtained from this initial precipitate by repeated recrystallisation as a very dilute solution (0·01 - 0·2%) from butanol-saturated water. Three or four such recrystallisations usually sufficed to yield an amylose with a 21% uptake of iodine as determined potentiometrically.

8) Superimposition of one of the preceding general methods upon another has been applied by some workers in attempts to obtain purer fractions. For example, Kerr and Severson (133,134) applied the butanol method to crude amylose solutions obtained from swollen corn and tapioca granules by aqueous leaching and thus isolated amylose samples which possessed "iodine adsorptions" of 20·5% and 20·7% respectively. In the case of corn amylose, the butanol-complex was obviously crystalline and assumed the form of rectangular platelets which retained their structure and crystalline properties even after removal of the butanol by washing with methanol and drying at low temperatures <u>in vacuo</u>. Tapioca and potato amyloses

isolated similarly assumed the form of radiating clusters of thick needles. Corn amylose could also be induced to crystallise as star-like clusters of thin needles by modification of the subfractionation technique (135). These products Kerr designated as "crystalline amyloses" (Compare Wiegel 136).

By first leaching out shorter-chain amyloses from maize and potato starch granules by means of hot water and then subjecting the leached granules to the precipitation technique, Meyer (137) has obtained amyloses consisting of about 2,100 glucose units, a chain-length very much in excess of the majority of amylose chains.

Certain workers (138,103) have claimed the removal by adsorption on cellulose of small proportions of amylose which persist in amylopectin preparations obtained by butanol etc. fractionations. Such reports were based upon determination of percentage iodine adsorptions of the amylopectin fractions. This value was found to be reduced after treatment with cellulose and further, the addition of a trace of iodine no longer gave a blue coloration. It was later shown (105,102), however, that separation of the contaminating amylose was not effected by this means but that the characteristic iodine reaction of amylose was merely masked by its adsorption of fatty impurities from the cellulosic material. When the supposedly

purified amylopectin was extracted with fat-removing solvents, it again gave a blue colour with iodine. Confirmatory evidence of the inefficacy of this technique was provided when identical percentage iodine adsorptions and iodine colorations were recorded before and after treatment with scoured cotton cellulose scrupulously freed from traces of fatty impurities.

To quote Higginbotham and Morrison (102), Dec., 1948, "no means of purifying amylopectin has yet been discovered, so the conditions of the initial precipitation must be chosen to keep the amount of amylose in the non-precipitated fraction as small as possible." However, in the special case of potato amylopectin with which almost all of the organic phosphate of the whole starch is associated, Hassid and McCready (94) reported further purification by the application of Samec's electrophoretic method to the crude fraction left as a residue after aqueous leaching of the swollen granules.

Amylose: Structure and Properties in Further Detail.

The blue coloration given with iodine was first recognised as a characteristic property of starch by de Claubry (139) in 1814. Attempts to account for the development of the blue colour have since ranged from speculations of definite compound formation with

the iodine (140) to those of adsorption (141) and of mere absorption of iodine. It is now realised that the amylose component, which is fundamentally responsible for the effect, takes up iodine by complex formation, while the association with amylopectin is explained on the basis of an absorptive or weakly adsorptive phenomenon. It is suggested that the amylose-iodine complex is formed by the long extended chains being induced to coil around the iodine molecules, the resulting complex thus assuming the shape of a helix.

Haworth (13) in 1929 suggested on purely geometrical grounds that α-linked glucopyranose units should be capable of such coiling. Hanes (142) later revived the idea and postulated a helical structure for starch and the starch-iodine complex. By enzymic degradation of starch he obtained a series of dextrins and showed that those containing less than six glucose units per molecule were not coloured by iodine. This fact was attributed to the inability of these dextrins to form one complete turn of a helix which Hanes suggested was necessary for iodine coloration.

Freudenberg (143) adopted and extended the above hypothesis and showed that it was possible to construct helical models (Stuart type, 144) of starch chains, each turn of the spiral being made up of six

glucopyranose residues. The internal dimensions of such helices were calculated to be of the right order of magnitude to neatly accommodate linearly arranged iodine molecules. It was pointed out that the interior linings of these helices would be essentially hydrocarbon in character and that the association of iodine molecules with such an "atmosphere" would be expected to affect the absorption of light in a manner similar to solutions of iodine in hydrocarbon or other nonpolar solvents. Since these solutions are characteristically blue to violet in colour, the analogy was cited to explain the blue colour of the starch-iodine complex. This hypothesis was opposed by Meyer (145) who contended that substances such as polyvinyl alcohol, cellulose swollen with zinc chloride and methylated cellulose, the molecules of which were known not to assume spiral configurations, also gave deep-blue colour-reactions with iodine. Attention was further directed to the fact that certain colloidal suspensions such as O-oxychalcone, acetocoumarin, and even inorganic compounds such as basic praeseodymium and lanthanum acetates all gave similar blue colorations with iodine. In these and other cases the effect is thought to be due to deposition of iodine within the fissures of colloidal precipitates which possess greatly distorted lattices, the blue colour arising by an accompanying change in

light absorption by the deposited iodine. Meyer suggested that the same or similar explanation applied to the starch-iodine colour, thus implying that a common mechanism must underlie the formation of all coloured iodine addition products. On the contrary, however, it seems unlikely that any one specific explanation would cover all the examples and the fact that a helical model fails to explain other blue products formed with iodine is no argument against its validity in the particular case of amylose.

The assumption of a helical model further serves to explain the formation of the two water-soluble, non-reducing dextrins (Schardinger dextrins, 147) produced by the action upon starch of an amylase from Bacillus macerans. It has been shown from X-ray data (148) that these dextrins consist of closed rings of six and seven glucose residues, the formation of which is thought to be facilitated by the screw-like arrangement of molecules from which they were derived. The first and sixth or seventh glucose residues in such spirals should be close enough together in space to allow them to react to form closed rings. Thus, according to Freudenberg the Schardinger dextrins do not exist preformed in starch. This view is supported by Kerr (149) who has shown that the Schardinger dextrins may be synthesised by the B. macerans enzyme from open-chain dextrins.

Bear (146) has pointed out, on the basis of X-ray evidence, that starch ordinarily exists in essentially linear, extended-chain configurations (e.g. in native granular starches and retrograded amylose (150)) and that it may only, under certain circumstances, assume the helical configuration (e.g. the amylose component induced to coil under the influence of such additives as iodine). The former type exhibit either "A" or "B" X-ray diffraction patterns (151,159) while the latter exhibit the "V"-type pattern (152,151,153,154).

It has long been known that the blue starch-iodine coloration may be discharged by bringing the solution to the boiling-point, but that on cooling the colour reappears (156). This phenomenon is now explained on the assumption that the application of heat tends to bring about extension of the coils with ultimate disruption of the helical structure and, therefore, of the amylose-iodine complex (134). Hence the disappearance of the blue colour. On recooling, however, the extended amylose chains again coil around the iodine molecules.

Apparently, selective precipitants of amylose such as n-butanol also aid the long, extended chains in assuming the helical configuration. Again, from the atomic model of Freudenberg it can be seen that the strongly polar hydroxyl groups should be located

on the exterior of the helical coils, and it has been suggested that these external hydroxyl groups are responsible for the crystallising properties of such complexes as that formed with n-butanol (134). X-ray investigations of butanol-precipitated amylose revealed excellent "V"-type diffraction patterns for this fraction either as the butanol complex or after removal of the butanol (154) or as the starch-iodine complex (153). These diffraction patterns confirmed a helical configuration with a helix diameter for the free amylose of about 13·7 Å , a length of turn of about 8 Å (i.e. the width of a glucose ring), and six glucose residues per turn. Other X-ray data (154) supported the suggestion that the iodine molecules of the starch-iodine complex constitute the cores of the helices. It has also been shown that the butanol and the fatty acid complexes (155) with amylose closely resemble in structure the iodine-amylose complex in that the complexing agents appear to occupy the interiors of the helices.

It is significant that amylose preparations exhibiting "V"-type diffraction patterns are capable of absorbing iodine vapour to form the complex in the absence of moisture or other substances. Yet on the other hand, amylose and starches in general which exhibit "A" or "B" diffraction patterns seem to require the presence of water and/or the iodide ion

for the production of the iodine complex (145). This seems to ascribe to water and the iodide ion an important part in helping the amylose to assume the necessary helical configuration which it lacks in the latter types of starch preparation (151). Dried butanol-precipitated amylose absorbs at least 26% of its own weight of iodine vapour and this corresponds to one iodine molecule per six anhydro-glucose units, that is, to one iodine molecule per turn of the helix (153). As mentioned previously, the highest reported value for iodine uptake as calculated from potentiometric data is 21·5% (104), but these determinations have necessarily been conducted in solution where the adsorption of other materials may impede that of iodine, e.g. the presence of iodide ions in solution impairs the uptake of free iodine by amylose (103), but as iodide ion concentration is reduced, percentage iodine adsorbed increases. From the results of their spectrophotometric titrations, Baldwin, Bear and Rundle (158) obtained a graph by plotting the number of glucose residues per iodine molecule adsorbed against a function of the iodide concentration and found that the curve extrapolated to the significant value of 6 glucose residues per iodine molecule at zero iodide concentration.

Rundle and Baldwin (157) reported that the adoption of a helical structure for the amylose-iodine

complex in which the long axes of the iodine molecules are coincident with the helix axis would be consistent with their observation of the distinct dichroism of flow exhibited by dilute amylose-iodine solutions. Their work was based on the discovery that light with its electric vector parallel to the long axis of an iodine molecule is strongly absorbed while light with its electric vector normal to the long axis of the molecule is only weakly absorbed. It was reasoned that by inducing a velocity gradient within solutions of the amylose-iodine complex, the proposed elongated helices should orientate themselves preferentially along the flow lines. Investigation of such solutions showed that light with its electric vector parallel to the flow lines (i.e. parallel to the long axes of the helical complex molecules) was more strongly absorbed than light with its electric vector normal to the flow lines (i.e. normal to the helical axes). This meant that the long axes of the iodine molecules combined in complex formation with the amylose were also orientated along the flow lines. This could be explained on the basis of any configuration in which the long axes of the iodine molecules and the long axis of the complex molecule were parallel. The observed dichroism of flow was therefore, at least in accordance with the helical structure outlined above for the amylose-iodine complex.

This technique also provided confirmation of the proposed differences in structure between amylose and amylopectin in that the unbranched, essentially linear amylose chains would be expected to orientate themselves more readily under the influence of a velocity gradient than the more highly ramified amylopectin molecules. This was, infact, found to be the case since the butanol-precipitated fractions of corn and potato starches stained with iodine displayed definite dichroism of flow while the non-precipitated fractions, as well as waxy maize starch and glycogen, each stained with iodine showed very weak or no dichroism of flow.

Further evidence in favour of the hypothesis that under certain conditions amylose possesses a helical configuration was provided by an examination of the optical properties of Kerr's "crystalline amylose" (150). When viewed on edge these platelets stained with iodine were found to be extremely dichroic in that light with its electric vector normal to the plane of the platelet was strongly absorbed while light with its electric vector parallel to the plane of the platelet was but weakly absorbed. This indicated that the long axes of the iodine molecules were normal to the largest face of the platelet. Furthermore, the unstained platelets were found to be quite birefringent when viewed on

edge indicating that the axes of the individual helices were also normal to the plane of the platelet. Again, these observations were interpreted in terms of a helical amylose chain, the complex with iodine being so constituted that the long axes of the iodine molecules were coincident with the long axis of the helix.

A suggestion as to the mechanism of the interaction of amylose and iodine resulting in complex formation has been advanced by Rundle, Foster and Baldwin (160). Their proposal is based on the possible possession by the amylose helix of a dipole moment parallel to the helix axis. According to the theory, an iodine molecule arranged parallel to the helix axis should acquire an induced dipole and thus exert an attractive influence on other similarly arranged iodine molecules in the vicinity.

For the determination of the chain-length of amylose samples, other physico-chemical, as well as purely chemical techniques were employed. On the basis of the yield of tetramethylglucopyranose (0·3 - 0·4%) obtained on hydrolysis of methylated maize and potato amyloses, Meyer and co-workers (125,126) estimated that, as an average, the chains consist of between 250 and 350 glucose units, and this conclusion received support from similar experiments conducted by Hess and Krajnc (161) and by Hassid and McCready (162).

The accuracy of this method of chain-length determination depends upon the absence of small proportions of amylopectin in the amylose samples under investigation since the yield of "end-group" obtained from methylated amylopectin is approximately ten times that obtained from methylated amylose. Another method for determining the chain-length of amylose is based upon its oxidation by the periodate ion, the mechanism of which is discussed later. By this means, the chain-length of potato amylose has been estimated to be of the order of some 500 glucose units (163). Other reports of the application of this method approximate the chain-lengths of potato and tapioca amyloses to 1000 glucose units while for wheat, corn, sago, Easter lily and apple amyloses, chain-lengths of between 400 and 600 have been calculated (164,183). The long straight-chain conception of the amylose molecule receives support from the fact that amylose triacetate readily forms strong films and fibres similar in properties to those given by cellulose triacetate (165). The property of retrogradation of amylose from aqueous solution is explained on the basis of orientation and aggregation of the long chains.

Meyer provided confirmation of the results of his "end-group" assay of the methylated products by estimations of the reducing end-group of the amylose molecule. He described two colorimetric techniques

designed for this purpose, the first involving the use of ammoniacal silver hydroxide, the intensity of the resulting colloidal suspension of silver being compared with the intensities of standard silver sols (125). The second method (166) was based upon the reduction of an alkaline solution of 3:5 dinitrosalicylic acid, the intensity of the colour developed being referred to a standard curve constructed by observing the colour intensities developed under identical conditions by maltose solutions over an appropriate range of concentrations. The results of these methods indicated D's P. of approximately 200 and 150-200 glucose residues respectively, for various amyloses. In this connection, it may be recalled that by using his modified "copper" technique, Richardson (44) estimated a D.P. of about 150 glucose units for water-soluble fractions of whole starches which he believed to be made up of unbranched chains of widely varying lengths. He proposed in 1936 that the shorter, more easily soluble chains constituted the amylose fraction.

In Meyer's opinion, most amylose chains are unbranched and he suggested that the differences between amyloses from various sources are due to differences in chain-length. This view is also held by Schoch (184). Foster and Hixon (167) also presented evidence based on viscosity data which favoured an essentially linear structure for various amyloses.

However, the possibility that some amyloses, at least, are slightly branched is not entirely excluded inasmuch as Hess and Steurer (168) found for an amylose sample a chain-length of some 240 glucose units as determined by "end-group" assay, but a D.P. of 650 glucose units calculated from osmotic pressure data. This corresponds roughly to three discrete chains per amylose molecule. Theoretically, the methylated product should yield about 0·4 - 0·5% of a dimethyl glucose on hydrolysis, but this would be extremely difficult to confirm by present techniques. Even if complete methylation of the amylose could be guaranteed, dimethyl glucose probably in excess of the above figure would still appear as an artefact due to partial demethylation, mainly of trisubstituted residues, by the acid used for hydrolysis. For example, Bell (169) recorded 2·2% demethylation during hydrolysis of 2:3:6 trimethylmethylglucoside by heating at 100° for 5 hours with a mixture of glacial acetic and 5% aqueous hydrochloric acids.

Kerr (134) suggested that the greater colloidal stability of potato amylose and particularly of tapioca amylose as compared with corn amylose is indicative of greater degrees of branching in the former structures. He bases further arguments in favour of this idea on the intrinsic viscosities of these amyloses as calculated from the data of Foster

and Hixon (167). Higginbotham (170) attributes the differences in the congealing tendencies of tapioca, maize and sago amyloses to variable degrees of slight branching in their structures. A strong argument in favour of non-branching of many amyloses is the fact that the limit of conversion with β-amylase approaches 100% (95,96), but Kerr explains this in the case of potato amylose by assuming that an 'anomaly' in the structure does not permanently block the activity of the enzyme, but merely reduces its rate of attack.

The synthesis in vitro of an amylose which is practically indistinguishable from amyloses isolated from natural whole starches was reported by Hanes (171) in 1940. He found that when glucose-1-phosphate (the Cori ester, 172) was incubated with plant phosphorylase (P-enzyme) a polyglucosan was deposited in the form of granules which closely resembled in appearance those of natural starches. This synthetic activity was found to proceed only in the presence of a carbohydrate activator or "primer" in the form of a pre-existent "template" chain of α 1:4-linked glucose residues, the function of which has been closely investigated recently by Peat and co-workers (173). The synthetic product was found to be sparingly soluble in hot water and to retrograde rapidly from solution. It gave a brilliant deep blue colour with iodine and

was found to be completely converted into maltose by the action of β-amylase. Hanes estimated its chain-length to be about 100 glucose units by determination of its reducing power. Confirmation of this value was provided by Haworth and co-workers (174) who applied the methylation technique to the problem and thus estimated a minimum chain-length of 80-90 glucose units; osmometric molecular weight determination of the methyl ether indicated the absence of branching in the chain (175). The main product of hydrolysis of the methylated material was shown to be 2:3:6 trimethyl glucose, and the "end-group" was identified as tetramethylglucopyranose. Other workers (176), however, were unable to detect any "end-group" amongst the products of hydrolysis of methylated "synthetic" amylose but their results have been criticised by Haworth (174). A "synthetic" amylose may also be obtained when glucose-1-phosphate is acted upon by muscle phosphorylase (177). The product was investigated by the methylation technique and the yield of "end-group" shown to correspond with a chain-length of 200-250 glucose residues.

Amylopectin: Structure and Properties in Further Detail.

The Haworth-Hirst 'laminated' formula originally proposed for whole starch suffers but slight modification in being applied to what is now recognised as the

major component of a natural mixture of differently constituted polysaccharides. Such modification pertains only to the length of the "unit-chain" for which compensation can readily be made in adapting it to the amylopectin molecule, by knowing the average chain-length of amylose and the proportion in which this component occurs in whole starches. Thus methylated whole starches were found to yield 4-5% of tetramethylglucopyranose on hydrolysis, and it is now known that the proportion of amylose in the majority of starches investigated was of the order of 20% and that hydrolysis of methylated amylose liberates some 0·3 - 0·4% of tetramethylglucose. It follows then, that the methylated amylopectin component must have been responsible for the liberation of between 4·5 and 6·0% of its own weight of "end-group" which corresponds roughly to a "unit-chain" of 20-25 glucose residues.

Confirmation of this deduction in the cases of potato and maize amylopectins was supplied by the experiments of Meyer and co-workers (125,126) who performed "end-group" assays on the substantially pure amylopectins and calculated that one non-reducing terminal unit occurs per 25 anhydroglucose residues. Similar experiments conducted by Hess and Krajnc (161) and by Hassid and McCready (162) provided support for this conclusion in the case of potato amylopectin, while, more recently, the amylopectin separated from

barley starch (182) has been shown to possess a "unit-chain" the lengths of which also conforms closely with the value quoted above.

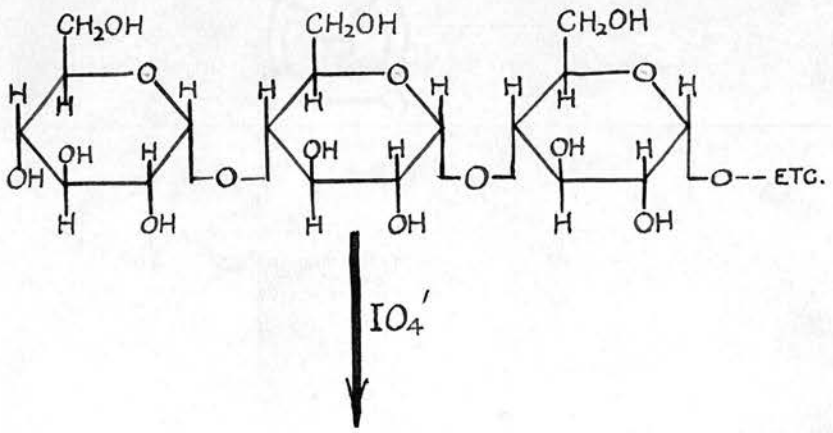
A novel technique for the determination of the proportion of non-reducing end-groups in starch, amylopectin and glycogen was introduced in 1945 by Hirst, Jones, et.al. (178,163,180). This method was based upon the fact that the periodate ion selectively oxidises α-glycols with characteristic cleavage of the C-C bond (181), according to the following equation,

$$R.CHOH.CHOH.R' + HIO_4 \longrightarrow R.CHO + R'CHO + HIO_3 + H_2O.$$

Compounds in which hydroxyl groups are attached to three contiguous carbon atoms are oxidised similarly, but with the liberation of formic acid, thus:-

$$R.CHOH.CHOH.CHOH.R' + 2HIO_4 \longrightarrow RCHO + R'CHO + H.COOH + 2HIO_3 + H_2O$$

Reference to the structural formula for portion of the starch chain (Fig.X) shows that although all the glucose residues thus represented are disposed to attack by the periodate ion, only the non-reducing terminal residues fulfil the conditions necessary for simultaneous liberation of formic acid.



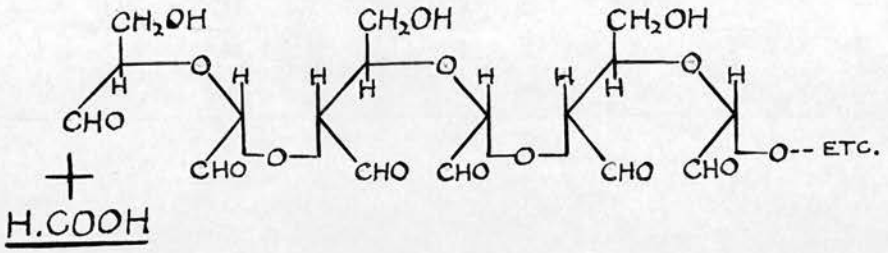



FIG. X.

Reducing end-groups are also susceptible to attack with the liberation of two moles of formic acid and one of formaldehyde according to the following scheme:-

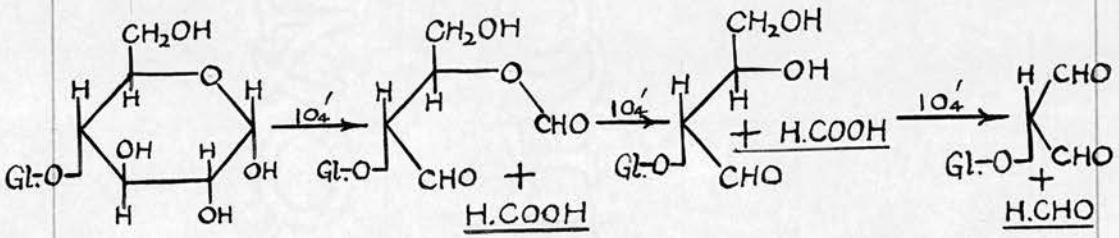



FIG. XI.

Now, the approximate mol.wgt. of amylopectin may be deduced by correcting for the relatively low average mol.wgt. of the amylose component in the high apparent mol.wgt. values estimated for whole starches during earlier work on the subject. Amylopectin mol. wgts. of three quarters to one million may thus be calculated and these values have received direct confirmation from osmotic pressure and viscosity data for the pure component. Hence, the undegraded amylopectin molecule contains some scores of non-reducing end-groups per single reducing end-group,

and the quantity of formic acid liberated from the latter by periodate oxidation, as compared with the total, may be regarded as negligible.

The method described by the above authors for the determination of non-reducing end-groups in starch and starch fractions involves oxidation of the polysaccharides at room temperature with an aqueous suspension of the sparingly soluble salt, potassium metaperiodate, which maintains the concentration of the periodate ion in solution at a very low value and thus prevents "over-oxidation" of the polysaccharides. Subsequent titration with dilute alkali (after addition of ethylene glycol) enables the calculation of the quantity of formic acid liberated during oxidation for 150 hours which is the time interval required for the liberation of 1 mole of formic acid from β-methyl-maltoside under similar conditions (179). Using the data thus obtained, the lengths of the unit-chains of several pure amylopectins were readily calculated. To facilitate calculation of the unit-chain lengths of the amylopectin components of whole starches, the quantity of formic acid liberated by the amylose components was neglected as a first approximation, provided that the amylose content did not exceed 25% and that the percentage of end-group in the amylose, reducing as well as non-reducing, was less than 1%. For the majority of starches examined these two

conditions were satisfied. The amylopectin contents of the starches were estimated by difference, having accurately determined the amylose contents by potentiometric iodine titration (103). Thus, on the assumption that the yield of formic acid was derived only from the non-reducing terminal residues of a known weight of amylopectin in the whole starches oxidised, the length of the unit-chain of this component could be calculated. Some results obtained by the use of this method are given in Table III. It is of interest to compare the values shown in column 2 of Table III with those for the corresponding starches listed in Table I which was compiled from methylation data.

| Source of Starch | (1) Percentage Amylose Content. | (2) Av.no.glucose residues per non-reducing end-group in whole starch calculated from $IO_4'$ ox. | (3) Calculated no.of glucose residues per unit-chain in amylopectin component. |
|---|---|---|---|
| Potato | 18 | 31 | 26 |
| Maize | 23 | 25 | 20 |
| Rice | 15 | 24 | 21 |
| Wheat | 19 | 26 | 21 |
| Banana | 21 | 27 | 21 |
| Sago | 26 | 24 | 18 |
| Amylopectins. | | | |
| Waxy Maize starch | 0-1 | 20 | 20 |
| Potato amylopectin | 0-1 | 24 | 24 |
| Barley " (182) | 2 | 24 | 24 |
| Apple " (183) | 0 | 24 | 24 |

TABLE III.

The development of this technique thus provided further evidence for a unit-chain length of 20-25 glucose residues for the amylopectin components of many of the more common varieties of starch. Obviously, the efficacy of the method depends upon the absence of glucose residues (other than non-reducing terminal residues) which could also liberate formic acid on periodate oxidation, e.g. residues linked solely through $C_1$ and $C_6$ positions, thus:-

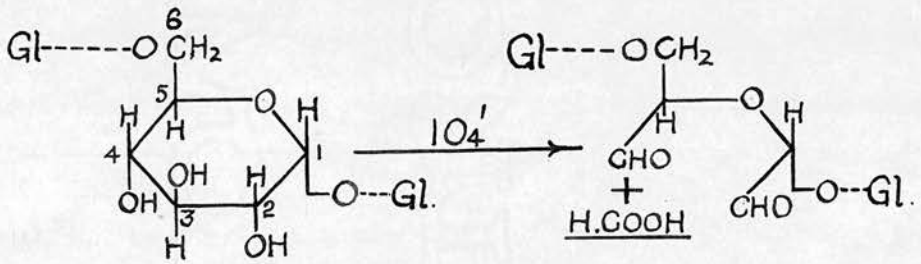
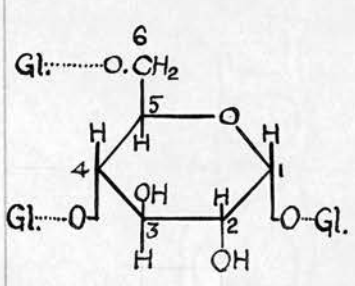



FIG. XII.

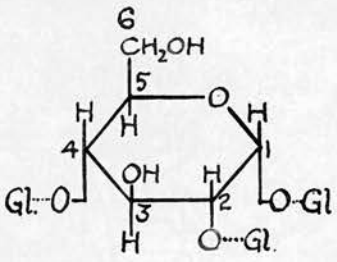
The absence of such residues both in amylopectin and in amylose was proved by the fact that the values for "average number of glucose residues per non-reducing end-group " calculated from both methylation and periodate oxidation data were identical within the limits of experimental error in the case of every whole starch investigated. From the results of methylation experiments Freudenberg (189) also concluded that 1:6 linkages without branching do not occur in starch.

Periodate oxidation has also proved of value in investigations on the nature of the inter-unit bonds in amylopectin (185). The evidence for the existence of 1:6 linkages in whole starch was reviewed in Part I of this chapter, and evidence was also outlined for the existence of at least one other glycosidic type of inter-unit bond (53,56,57). Since amylose has been shown to be an essentially straight-chain α 1:4-linked polymer, it follows that this evidence now pertains to the amylopectin component. Apart from inter-unit bonding through 1:6 links those through the 1:3 and 1:2 positions are also possible. Three structurally different tri-substituted glucose residues may, therefore, be responsible for branching in the amylopectin molecule; they are represented in the following formulae:



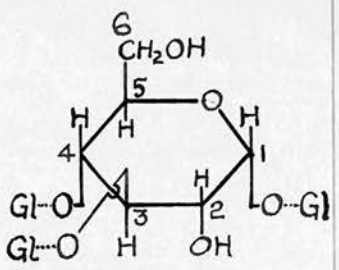

SIDE-CHAIN ATTACHED THROUGH $C_6$.

Fig. XIII.




SIDE-CHAIN ATTACHED THROUGH $C_2$.

Fig. XIV.




SIDE-CHAIN ATTACHED THROUGH $C_3$.

Fig. XV.

Inspection of the structure represented by Fig. XIII reveals two free hydroxyl groups attached to

adjacent carbon atoms 2 and 3. Such a glucose residue is oxidisable by the periodate ion with disruption of the $C_2$-$C_3$ bond. On the other hand, the glucose residues represented by Figs. XIV and XV would not be attacked by the periodate ion since they do not contain free hydroxyl groups attached to contiguous carbon atoms. This means that of all glucose residues in the amylopectin molecule, only those which are responsible for possible inter-unit bonding through carbon atoms 2 and 3 should remain intact after periodate oxidation, and thus be recoverable as glucose after hydrolysis of the oxidised polysaccharide. In the cases of potato, acorn, sago and waxy maize starches less than 1% of glucose by weight of the starch used was thus recovered and estimated by the method of quantitative paper chromatography (186). The quantitative significance of such a small proportion of glucose is doubtful. It could, for example, have arisen through incomplete oxidation due to steric hindrance by the ramified amylopectin molecule rather than from residues structurally immune to attack by periodate ion. Irrespective of the actual source of the recovered glucose, however, this investigation provided clear evidence that more than 75% of the inter-unit bonds of the amylopectins studied were of the 1:6 variety and that a relatively small proportion only of the inter-unit bonds could engage hydroxyl

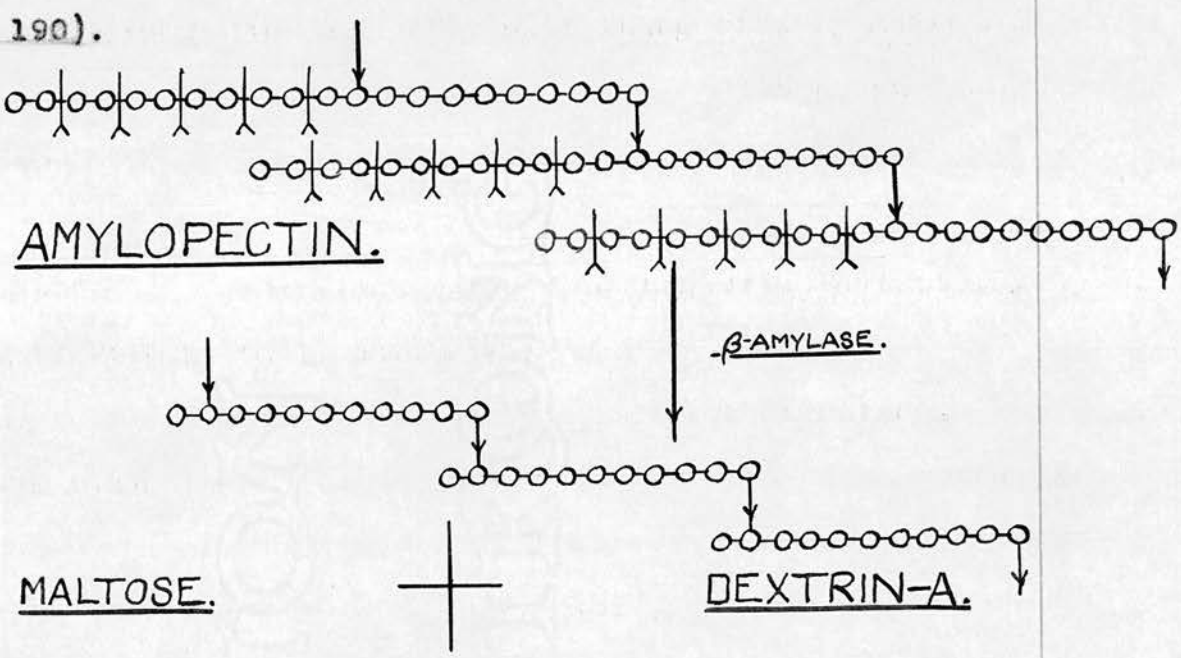
groups attached to either $C_2$ or $C_3$ of glucose residues in the unit-chains.

As some uncertainty exists as to the influence exerted by methylated amylose upon the course of 'disaggregations' conducted in earlier experiments on methylated whole starches, Halsall, Hirst and Jones (187) repeated the investigation using methylated waxy maize starch. The velocity constant and activation energy for the reaction were found to be of the same order as those previously quoted for methylated whole starches (52), thus indicating again that covalent bonds were being ruptured. The limiting viscosity of the products of disaggregation of this methylated amylopectin corresponded with a mol.wgt. of approximately 20,000 (i.e. a D.P. of 90-100 glucose residues), a value agreeing closely with that determined for disaggregated whole starches. Since the 1:4 links of the unit-chains remain intact during disaggregation and since waxy maize starch possesses a unit-chain consisting of about 20 glucose residues, it follows that the fully disaggregated polysaccharide molecules consisted, on the average, of five of these unit-chains. Such aggregates of unit-chains (possibly all inter-united through 1:6 links) may be regarded as the true repeating-units of the amylopectin molecule. They, in turn, apparently are joined by relatively acid-labile glycosidic linkages which, from this evidence,

appear to constitute some 20% of all the inter-unit bonds in the macro-molecule. It was further shown, by means of the periodate oxidation technique, that at least 80% of the inter-unit bonds of waxy maize starch are of the 1:6 variety, and by the arguments outlined above, the possibility exists that the remaining 20% may be 1:3 and/or 1:2 linkages. Accepting this evidence, it was suggested that it may be possible to identify such linkages with the 20% of inter-unit bonds broken during disaggregation. To account for the comparative ease with which these bonds are disrupted, it was further postulated that the glucose residues at the ends of appropriate unit-chains may be furanose and attached through their reducing groups to $C_2$ or $C_3$ of glucopyranose residues in adjacent chains. Substantial evidence for this suggestion, however, is not yet available.

Further to the subject of amylolysis of starch, the current theory of the so-called "sensitisation" of the limit dextrin-A by contact with salivary α-amylase amy now be outlined (188). Inasmuch as the amylose component of whole starches is completely convertible to maltose by β-amylase (95,96), it follows that the limit dextrin-A isolated after β-amylolysis of whole starches must arise from the amylopectin component. Fig.XVI illustrates the proposed mode of attack of β-amylase upon the unit-chains of the

amylopectin molecules by progressive removal of maltose units, one at a time, starting at the non-reducing terminal residues of the unit-chains (74,70, 190).




where "-O-" represents a glucopyranose residue linked α 1:4

and "⊥" represents points of attack by β-amylase.

FIG. XVI.

β-Amylolysis proceeds until 50-55% of the amylopectin has been converted into maltose (98,162), the residual structure (dextrin-A) being resistant to further attack by β-amylase the activity of which is thought to be obstructed by the "anomalous" cross-linkages between the unit-chains. It appears both from the results of methylation (72) and of periodate oxidation (192) that dextrin-A (from several varieties of amylopectin) contains one non-reducing terminal group

per 10-12 glucose residues. The fact that tetra-methylglucopyranose can be isolated from the hydrolysate of methylated dextrin-A suggests that β-amylase activity does not proceed completely to the glucose residue responsible for branching, but that it ceases at least one glucose residue from it, as indicated in the above diagram. Apparently, the mere proximity of the branching point is sufficient to inhibit the further activity of the enzyme.

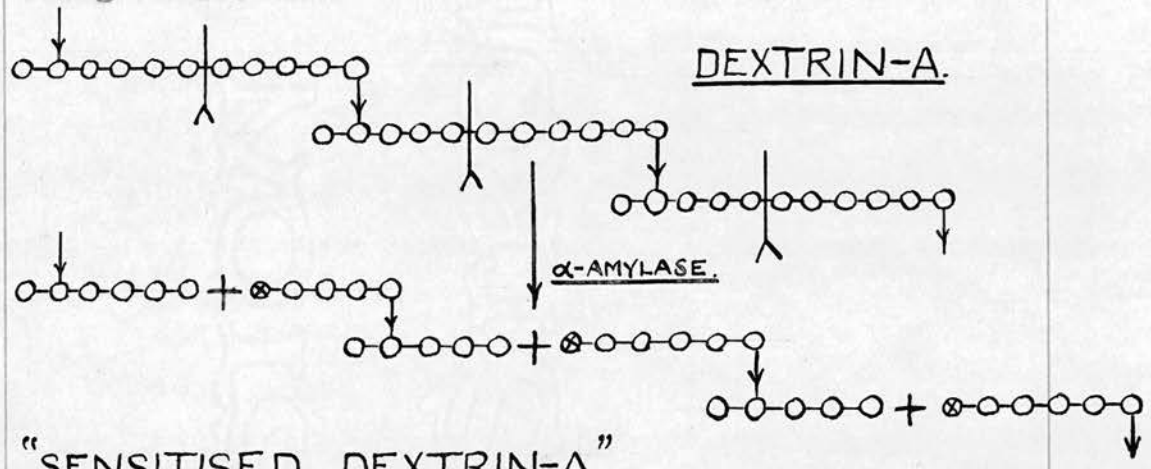
Relative to the exact location of these branching points within the unit-chains, it is of interest to note that, on the basis of the above hypothesis, a 50-55% conversion of amylopectin to maltose means that, as an average, slightly more than half of the glucose residues of the unit-chains separate the branching points from the non-reducing terminal residues.

If dextrin-A is submitted to the action of salivary α-amylase, it is said to be "sensitised" which means that it is rendered susceptible to further attack by β-amylase by means of which maltose is again produced along with a second limit dextrin known as dextrin-B. The quantitative aspect of these conversions was investigated and the results indicated that the 1:6 cross-linkages which obstruct the action of β-amylase are not hydrolysed during "sensitisation" of dextrin-A by α-amylase, a point which is contrary to earlier ideas on the subject (73). It is now

thought that the hydrolytic activity of both α- and β-amylases is confined to the normal 1:4 glycosidic linkages of the unit-chains, but that their mode of attack is quite different. Whereas β-amylase makes a systematic end-wise attack on the chains with separation of maltose units until a branching point is approached, α-amylase is apparently able to effect more or less indiscriminate hydrolytic scission of 1:4 linkages at almost any part of the unit-chains, regardless of whether or not they are situated between two branching points. Such a method of attack by α-amylase upon whole starch would account for the rapid fragmentation of the starch molecules into dextrins of relatively low mol.wgt., thereby liquefying the starch paste and destroying its iodine-colouring ability, as well as increasing its susceptibility to the action of β-amylase.

These views coincide with those expressed by Myrbäck (76) who suggested, however, that the linkages near to the end-groups and to the branching points of amylopectin possess a certain degree of protection against the initial activity particularly of malt α-amylase which should, therefore, first attack parts of the chains which are roughly equidistant from these points. By the same reasoning, the chains of dextrin-A should be ruptured preferentially at points situated centrally between the two branching points.

The "sensitisation" of dextrin-A is explained on this basis and from Fig.XVII it can be seen how α-amylase could thus liberate new "outer" chains with non-reducing terminal residues at which β-amylase could again begin its characteristic end-wise attack which would continue until a branching-point is again encountered, the residual structure in this case being dextrin-B.




"⊗" represent new non-reducing end-groups exposed to further attack by β-amylase.

FIG. XVII.

"Sensitisation" of dextrin-A may also be effected by treatment with dilute acids (76) or by heating in an autoclave with water at 120° (70).

Recently, Peat and co-workers (191) have isolated an enzyme (R-enzyme) which is thought to effect debranching of dextrin-A and of amylopectin by an irreversible mechanism. The activity of R-enzyme is

thought to be confined to hydrolytic scission of the 1:6 cross-linkages, the products of the reaction displaying marked increases in blue-value and susceptibility to attack by β-amylase as compared with those of the original substrate. However, 100% β-amylolysis of R-enzyme-treated dextrin-A and amylopectin was not achieved and a possible reason for this is considered to be the occurrence of cross-linkages other than those of the 1:6 variety. These other cross-linkages, not being attacked by R-enzyme, would persist in the products of its activity on dextrin-A and amylopectin, and thus create impediments to the subsequent action of β-amylase.

Dillon (193) reported the presence in preparations of wheat β-amylase of an enzyme which hydrolyses 1:3 linkages such as occur in laminarin and floridean starch. An argument was advanced for the presence of such linkages in amylopectin and was based upon the report of Hirst and co-workers (192) that limit dextrins produced from amylopectins by crystalline β-amylase possess much higher mol.wgts. than those from the crude wheat enzyme.

The facts relating to the amylolysis of amylopectin have been discussed on the basis of the Haworth-Hirst 'laminated' structure which represents the simplest formula yet proposed which is consistent with the known facts. A variation of this formula has been

adopted by Meyer (98)(89) to explain the results of his enzymolysis experiments. He visualises an irregular model with multiple, random "maple-tree" branching, sometimes called the "net" structure (Fig. XVIII). Myrbäck (76) considers that this formula offers a satisfactory explanation of the action of amylases and further supports it on the grounds of calculations based on a possible mechanism of synthesis by enzymes.

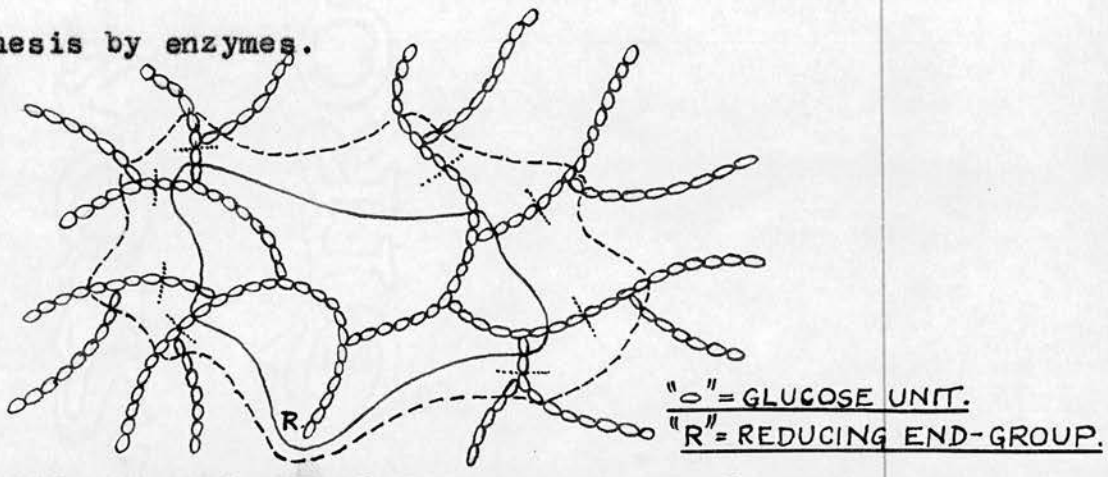



Fig.XVIII. Meyer's "Net" Structure for Amylopectin (1940)

On the basis of such a model for amylopectin, the structure of limit dextrin-A (called 'Grenzdextrin I' by Meyer) should resemble that enclosed by the broken lines in the figure. This boundary represents the limit of the initial attack by β-amylase. Meyer found that dextrin-A could be "sensitised" by contact with α-glucosidase (yeast maltase) which splits off glucose units from the ends of the chains, hydrolysis continuing to positions such as those marked by the short dotted lines in the figure. The fact that

α-glucosidase but not β-glucosidase (emulsin) brings about this hydrolysis is taken as evidence of the α-configuration of the branching linkages. β-Amylase could again act on the residual structure starting at the newly-exposed non-reducing end-groups until its progress is again impeded by encounter with further branching-points, the products in this case being maltose and 'Grenzdextrin III.'

An attempt was made to distinguish between the highly ramified and the 'laminated' structures by examining the properties of dextrin-A (194). Meyer reasoned that the 'laminated' structure should give rise to an elongated, rod-shaped molecule for this dextrin which, on the other hand, should be more compact and globular in form if derived from the branched structure. Films obtained on evaporation of solutions of acetylated dextrin-A were found to be extremely brittle and this property was regarded as evidence in favour of the branched structure for the parent amylopectin. In reply to Meyer's communication, Haworth (195) stated that he considered this evidence too slender to support Meyer's contention and that the multi-branched structure seemed premature and unnecessarily complex in the absence of a more complete experimental background. Evidence against Meyer's views on the structure of amylopectin based on a comparison of the specific viscosities of the limit

dextrins obtained from starch and glycogen was presented by Hirst, et.al. (192) who suggested that the observed facts favoured a more spherical shape for glycogen in contrast to a linear type of structure for amylopectin and its limit dextrin to which the Staudinger viscosity relationships have been found to be applicable.

A multi-branched formulation is quite consistent with the results of the end-group assay since this method depends upon the overall relative proportions of tetramethyl to trimethyl glucose and, therefore, does not distinguish between uniformly and randomly branched structures. On the other hand, however, it is difficult to reconcile the results of the disaggregation experiments with an irregular multi-branched structure from which one would not expect to obtain the homogeneous disaggregation product which is, in fact, actually derived from amylopectin.

Certain workers consider the prevailing dualistic concept of starch constitution to be of minor importance and do not agree that the linear amylose component represents a true entity co-existing with the branching amylopectin component. Pacsu, for example, prefers to regard amylose as an artifact formed during gelatinisation or complete disruption of the granules during fractionation procedures. He suggests that the visible starch granule may be a

three-dimensional network of glucose anhydride units held together by regular α 1:4 glycosidic bonds and, at short but definite intervals, by acetal or hemi-acetal bonds originating from open-chain glucose anhydride residues (196). The results of periodate titration, however, eliminate the possibility of hemi-acetal bonding since the glucose residues responsible for such bonding would contain hydroxyl groups attached to three contiguous carbon atoms as depicted in Fig.XIX (163, 197). Inasmuch as these glucose residues would not be non-reducing terminal residues, the yield of formic acid obtained on periodate oxidation would be in excess of that which could be accounted for by the latter. As explained previously, the yield of formic acid actually obtained on periodate oxidation of starches coincides with the theoretical yield calculated on the basis of the predetermined proportion of non-reducing terminal residues only (163). Therefore, residues such as that illustrated in Fig.XIX must be absent. But this argument does not eliminate glucose residues containing acetal bonds as in the following variation of Pacsu's

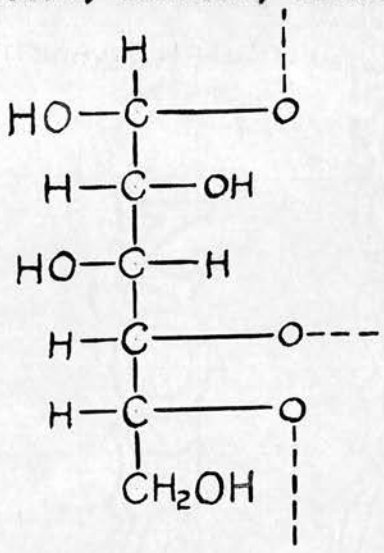


FIG. XIX.

formula (Fig.XX) which illustrates the essential features of his conception of the starch molecule.

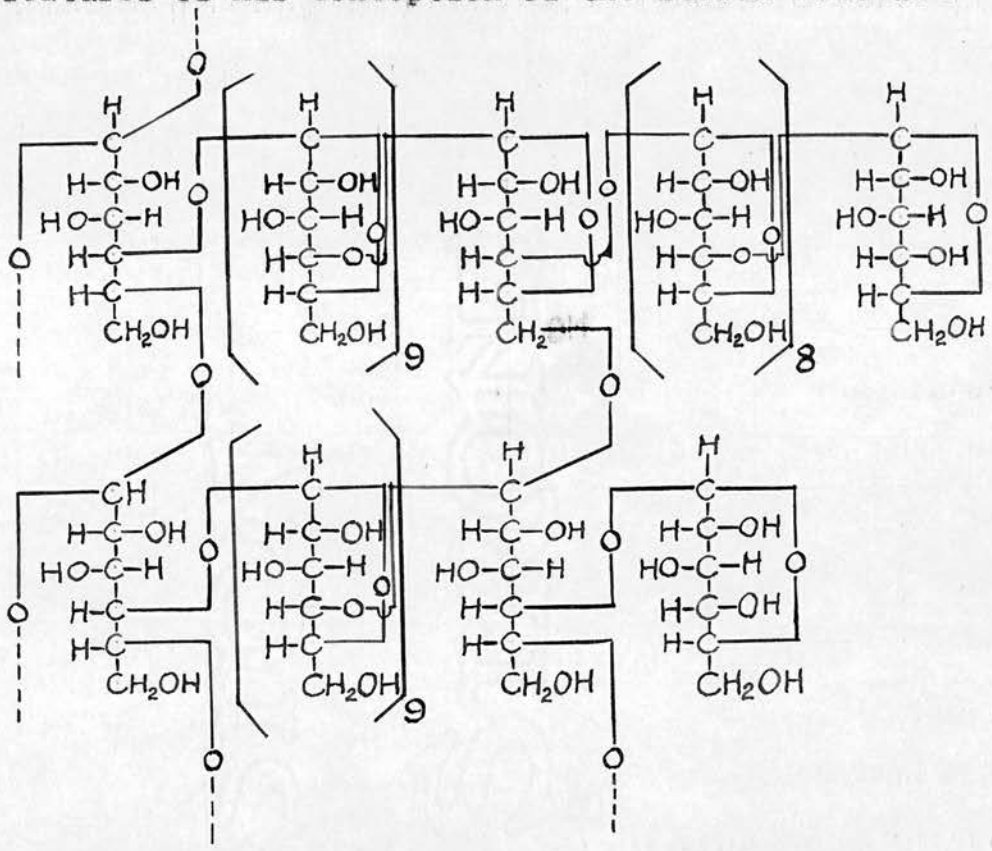


FIG.XX. Pacsu's Formula for Starch (1946).

The rapid initial degradation of starch on mild acid-catalysed hydrolysis is explained by the presence of acetal bonds which are known to be extremely susceptible to acidic hydrolysis, and in Pacsu's opinion such bonds are identical with those ruptured during disaggregation. According to this worker, the rapid and complete scission of appropriate acetal bonds during mild acid treatment could give rise to straight-chain structures with properties resembling those of amylose and he claims to have

isolated an amylose fraction from waxy maize starch by such treatment. He proposes that amyloses of other starches arise in a similar fashion. These views are opposed by the results of potentiometric iodine titrations carried out under identical conditions on a number of starches including the 'waxy' starches, the properties of which are shown to differ quite distinctly from those of other starches (103). Also, if Pacsu's views are correct, it is difficult to see why, under identical conditions of fractionation, most starches should yield amylose in appreciable quantities while the 'waxy' varieties yield, at most, less than 2% of amylose (129,198).

Enzymic Synthesis of Amylopectin.

An important aspect of starch chemistry which has received considerable attention during recent years is the enzymic synthesis in vitro of both amylose and amylopectin. The isolation by Hanes (171) of the amylose-synthesising phosphorylase (P-enzyme) was followed by the isolation and purification by Peat and co-workers (175,199) of another enzyme (called Q-enzyme) which also occurs in potato juice. Q-enzyme was also found to possess both hydrolytic and synthetic powers, the latter activity resulting in the formation of amylopectin, but it was shown that for the fulfilment of its synthetic function, Q-enzyme must be

provided with a suitable substrate. This substrate has been called "pseudo-amylose" (200) and is thought to be constituted as single, unbranched chains each containing about 20 glucose units mutually linked by α 1:4 glycosidic bonds. Pseudo-amylose may be formed either by the synthetic activity of P-enzyme upon glucose-1-phosphate or by the hydrolytic activity of Q-enzyme upon amylose (200). Unlike P-enzyme, Q-enzyme does not appear to act upon amylose phosphorolytically, that is, it does not appear to bring about hydrolysis through the agency of $HPO_4''$ ions (201). The hydrolytic function of Q-enzyme thus identifies it as an amylase, but a study of its properties shows that it cannot be classified simply either as an α- or a β-amylase (202).

It is thought that pseudo-amylose is the only substrate upon which Q-enzyme can act synthetically to produce the branched-chain structure of amylopectin, presumably by the formation of 1:6 cross-linkages between the pseudo-amylose chains. It seems probable that in the conversion of amylose to amylopectin by Q-enzyme that this cross-linking occurs simultaneously with each hydrolytic scission of a 1:4 link in the amylose chain and that no appreciable quantity of the short unbranched chains of pseudo-amylose is present at any time (203).

"Synthetic" amylopectin possesses a unit-chain of

20 glucose residues, gives a red-purple colour with iodine, is easily soluble to form stable solutions and is converted to maltose by β-amylase to the extent of about 50% only (200). In these properties, it is indistinguishable from amylopectins from natural sources. It has a D.P. of about 100 glucose units only and in this regard differs from the natural amylopectins which possess much larger molecules (175).

The following scheme illustrates the various functions of P- and Q-enzymes and shows the mechanism of formation of amylopectin from glucose-1-phosphate.

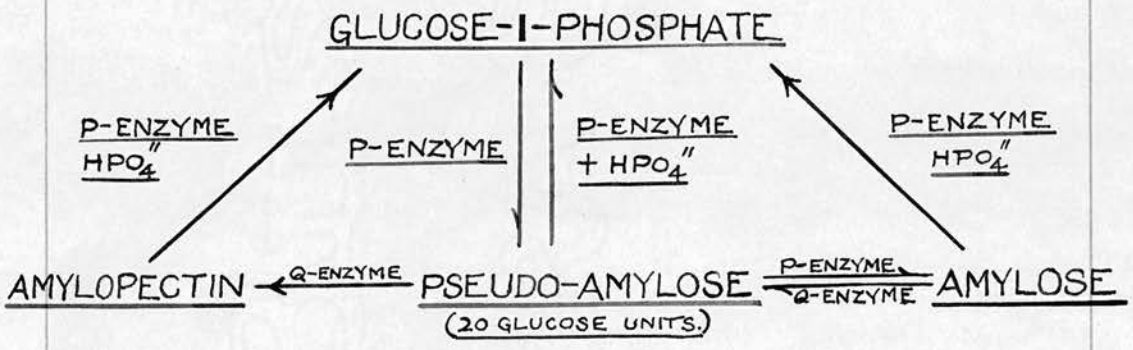



FIG. XXI.

## CHAPTER II.

## D I S C U S S I O N.

Chemical investigations upon the constitution of starch have centred mainly about those varieties which occur in nature in greatest abundance and which are most readily accessible, namely, the starches of the grass-like plants (cereal starches) and those of roots and tubers, especially of potatoes. The majority of starches so far examined have been found to contain 15-25% of amylose, but the 'waxy' starches which contain only traces of amylose and the starch from the "Steadfast" variety of wrinkled pea, which at the other extreme, consists almost entirely of amylose, provide marked exceptions to the general rule (103,106). Reference to Table III shows, furthermore, that the lengths of the unit-chains of the amylopectin components of the several starches listed vary over an appreciable range (163). A similar variation has been observed in the chemically-related glycogen series in which the size of the repeating-units of certain samples of rabbit-liver glycogen has been found to deviate conspicuously from that of many other glycogens (178,204). It seems important, therefore, in extended investigations upon the constitution of starch, to examine samples from yet other botanical sources to ascertain how their structures and properties

compare with those of the starches already studied. Hence the present contribution which reports the results of an investigation of starches obtained from the sapwoods of several species of trees.

The samples were kindly supplied by Mr. W.G. Campbell of the Forest Products Research Section of the D.S.I.R. and were isolated in granular form by Mr. Campbell (234) in the following way. The sapwood of selected logs was converted to sawdust which was vigorously mechanically stirred with cold water for about one hour. The resulting suspension was filtered to remove wood particles using a Jena G1 or G2 sintered glass filter, and the starch grains then allowed to settle out from the filtrate. The grains were freed from remaining small particles of wood by a process of fractional sedimentation, the wood fibres settling from aqueous medium more rapidly than the starch grains. The final suspension was allowed to settle completely and the supernatant liquid decanted off. The residual wood-starch granules were then washed with cold acetone until the solvent removed no further colouring matter, after which they were collected on a silk filter, washed with ether and air-dried. The yields of starch thus obtained from maple and elm sapwoods, namely 0·075% and 0·16% respectively, are roughly typical of those from other sapwoods, but this method of separation of starch grains from wood was

not claimed to be in any way quantitative. The total starch contents of the sapwoods of the common timber species have been estimated by several investigators (205), and the following representative examples may be quoted, the values being expressed as percentages of the dry weight of the various woods: maple 2·65, elm 5·9, oak 2·1, sycamore 4·7, ash 2·4, beech 0·2, and horse-chestnut 6·7%.

In the search for suitable sources of wood-starch account has to be taken of the well-defined seasonal variation of starch in the living tree (205). It has been well established that for deciduous trees in temperate climates there is a maximum of starch in the trunk at leaf-fall and a secondary maximum in early spring, while corresponding minima occur during the periods of dormancy and growth. The autumn maximum is the cumulative result of photosynthetic activity throughout the preceding summer, and the spring depletion corresponds with fresh growth of leaf and shoot, the starch being converted to soluble sugars which are then transported to positions where they are required for transformation to cellulose and other products. It is thought that the other fluctuations are the manifestation of a protective mechanism against the action of frost on the living cells rather than a result of the actual growth rhythm of the tree (206).

Although the isolation of wood-starch for chemical study was first accomplished by Campbell (207) in 1933, most of the work on this starch has been concerned with the commercially important problem of depleting its content in timber with a view to rendering the timber immune to attack by wood-destroying organisms. It is now well known that starch can be utilised as food by the larvae of Lyctus powder-post beetles and that starch-free sapwood is an unsuitable medium for the development of these insects (208).

The starches with which we were provided were isolated from the sapwoods of elm, maple, oak and turkey-oak logs, the samples ranging in colour from brown to pale buff. Microscopic examination revealed that the granules of all four samples are of similar dimensions, the larger grains measuring up to 10-12 $\mu$ along the longer axes. The granules of wood-starches are thus comparable in size with those of rice and buckwheat which are among the smallest of the many known varieties. Starch granules from canna and potato are among the largest, measuring up to 145 $\mu$ and 100 $\mu$, respectively (209). The shape, as well as the size, of starch granules is characteristic of their botanical source. Wood-starch granules, particularly those from elm sapwood, may be characterised by their great variability in shape which ranges from roughly cubic and tetrahedral to

spherical and even to dumb-bell shaped. They possess well-defined concentric striations about a well-defined hilum some of which resemble the bifurcated hilum of leguminuous starch granules.

It has been reported that wood-starch granules possess no apparent granular membrane, in which case, they are unique (210). The granules of all the known starches, on heating in aqueous medium, can be seen to swell suddenly at a certain definite temperature known as the 'gelation point' which varies somewhat from variety to variety of starch and according to its pre-treatment. This sudden swelling is accompanied soon after by the apparent bursting of what seems to be a surrounding pellicle or membrane, and under favourable microscopic conditions, the fluid contents of certain of the distended granules can be seen to be ejected suddenly and to stream out into the aqueous medium, leaving ruptured sacs which soon fall to crumpled, shapeless masses. This effect is comparatively easily observed with the larger granule species, but as the granule size decreases to that of the wood-starches it is reasonable to assume that greater difficulty would be experienced. The author considers that observation of wood-starch granules under the following conditions facilitates the detection of what appears to be a granular membrane or pellicle which is similar in form to those

observable under the same conditions with potato and barley starch granules. A little of each sample was dusted separately on to a microscope slide and well covered with water. A cover-slip was gently pressed over the suspension and the slide then placed on the microscope stage electrically heated previously to 50°. Heating was continued at the rate of 1° per minute during which process the granules were microscopically observed at a magnification of 500. In the case of elm sapwood starch, the majority of granules swelled suddenly over the range 54-55° to approximately 10-15 times their original size and then appeared to burst leaving ruptured sac-like objects which did not disappear even on heating to 70°, but which tended to clump together to form a reticulum. It was observed that the granules suffered sudden and almost complete loss of opacity at the point of 'bursting' although their outlines remained sharply enough defined. The other wood-starch samples behaved similarly and their respective gelation points were found to be as follows: maple starch 54-55°, English oak 55-56°, turkey oak 56-57°. For comparison, the gelation point of a sample of potato starch was determined under identical conditions and was found to be 61·5 - 62·5°.

With one exception, weak pastes of each wood starch with water gave with iodine solution a blue

coloration which was discharged on heating above 80°, but which reappeared on cooling. The exceptional sample which gave a reddish-blue colour with iodine solution had been bleached by the passage of chlorine through an aqueous suspension of the granules followed by acidification with sulphurous acid for the destruction of excess chlorine. Evidence is presented which strongly suggests that this treatment has brought about considerable degradation of this starch sample. Fortunately, however, a small quantity of an undegraded sample of oak sapwood starch was also available for study.

As mentioned previously, the granules of the various samples range in colour from brown to pale buff due to the adsorption of coloured impurities either during their formation and deposition within the living sapwood cells or, more probably, during their aqueous extraction from the sawdusts. Besides varying amounts of fibrous material, the impurities probably include anthocyanin(s), lignin and gallotannin of which the latter has proved to be the most troublesome. The various tests which were applied for the detection of these impurities are given in the experimental section. Difficulty was experienced in removing the impurities without simultaneous degradation of the starches, although methylation of elm sapwood starch by repeated treatment with dimethyl

sulphate and sodium hydroxide was found to effect progressive removal of the coloured impurities, yielding finally a pure white derivative possessing relatively high solution viscosity. The impurities were thus found to be very strongly adsorbed on the carbohydrate lattice, the conclusion that lignin and gallotannin are, in fact, merely adsorbed incidental impurities, at least on elm starch granules, being arrived at by inspection of ultraviolet absorption photographs kindly prepared for us by Professor H. Erdtman and Dr. P Lange of the Royal Institute of Technology, Stockholm. Lignin and tannins strongly absorb ultraviolet light of wave-length about 2800 Å and the photomicrographs revealed that some of the starch granules do not absorb in this region while those which do absorb vary in the degree to which they do so. Apparently, therefore, the granules are unevenly contaminated with the above impurities, but it is difficult to decide whether these completely impregnate or merely encrust the granules.

Complications to several of the various standard analytical procedures normally applied to starches were introduced by the gallotannin impurity of the wood-starch samples. For example, the procedure prescribed for the potentiometric iodine titration of starches for the assay of amylose contents (103) had to be modified slightly in being applied to the

gallotannin-contaminated wood starches, for the following reason. Normally, the starch sample (ca. 0·04 g.) to be analysed is thoroughly dispersed in potassium hydroxide (10 ml.; 0·5N) which, according to the amendment of Wilson, Schoch and Hudson (128) to the original procedure, is then neutralised to methyl orange by the addition of hydrochloric acid (0·5N). Potassium iodide (10 ml.; 0·5N) is then added for the subsequent establishment of the iodine-iodide electrode, and the volume made up to 100 ml. with distilled water. A shiny platinum electrode and a saturated calomel cell are then inserted into the solution and connected appropriately to a potentiometer by means of which the E.M.F. of the system is determined after each addition of iodine solution (1 ml.; 0·001N in 0·05N potassium iodide). Obviously, the accuracy of the analysis depends upon knowing exactly the quantities of iodine progressively being added. In the case of the wood starch samples, however, the preliminary dispersion in potassium hydroxide solution was accompanied by atmospheric oxidation of the gallotannin impurity to ellagic acid. After neutralisation of the dispersions and addition of the potassium iodide, it was observed that the solutions soon became pale purple in colour and that during the next hour the intensity of this coloration slowly increased. This effect was found to be due to

the gradual liberation of free iodine, presumably by oxidation of potassium iodide by the ellagic acid, the liberated iodine then, of course, reacting in characteristic fashion to form the starch-iodine complex with the dispersed wood starch. In preparing the starch dispersions for the actual potentiometric iodine titrations it was essential to remove this liberated iodine. This was, in effect, accomplished by the addition of dilute thiosulphate solution until the dispersions lost their purple coloration and resumed their original brownish shade. Owing to this latter inherent colour of wood starch dispersions in aqueous medium it was desirable to facilitate detection of this 'end-point' by the use of a 'control' dispersion of similar concentration to which potassium iodide had not been added and with which, therefore, the colour of the experimental dispersion could reliably be matched. In this way, it was found that certain 40 mg. dispersions required as much as 12 ml. of sodium thiosulphate solution (0·001N)to reach an 'end-point.' Potentiometric detection of this 'end-point' was also attempted, and titration curves were prepared by plotting "measured E.M.F." against "volume of thiosulphate solution added." Although these curves showed typical and reasonably good end-points, this technique was considered to be no more accurate than the above colorimetric method, and it was certainly

not as convenient. After the removal of the 'free' iodine from each of the starch dispersions, the volumes were made up to 100 mls. with water, and the potentiometric iodine titrations then conducted in the normal manner.

The efficacy of this procedure in providing results of the desired degree of accuracy was demonstrated by adding a little of an authentic sample of gallotannin to a sample of barley starch the amylose content of which had previously been estimated by potentiometric iodine titration as 18·7% (182). The contaminated barley starch was treated as described above for wood starches and the same effect of slow liberation of free iodine after addition of the potassium iodide was observed. This served to confirm that gallotannin impurity was, in fact, primarily responsible for this effect in the case of the wood starches. Potentiometric iodine titration of the contaminated barley starch sample after 'removal' of the liberated iodine gave a value for the amylose content of 18·6% which is identical within the limits of experimental error with that quoted above for the pure barley starch.

In the first two iodometric analyses of elm starch the oxidative liberation of free iodine within the dispersions was ignored, that is, the potentiometric titrations were conducted without the preliminary

'removal' of this incidental free iodine. Although the results, in these cases, were thus rendered meaningless, they are included here in the form of graphs (FIG.XXII) to illustrate the effect of gallotannin impurity on the course of the titration and, therefore, upon the value for "apparent amylose content" of elm starch. Comparison of graphs I and II (obtained by plotting "percentage iodine uptake" against "free iodine concentration,") reveals that this value is conditioned by the length of the time interval which elapsed between the addition of the potassium iodide solution to the neutralised dispersion and the beginning of the actual potentiometric titration. The inflection point of graph I which represents the potentiometric iodine titration of elm starch (20 mg.) started 15 minutes after the addition of the potassium iodide, corresponds with an amylose content of 10·2%, while the inflection point of graph II, representing a titration started 25 minutes after the addition of the potassium iodide, corresponds with an amylose content of 7·2%. The reason for the difference between the results of these two attempted analyses is that the liberation of iodine from the potassium iodide continued slowly in each case and was probably only complete approximately one hour after the addition of this reagent to the respective dispersions. Hence, the dispersion on which the

titration was started 15 minutes after the addition of potassium iodide required the addition of more standard iodine solution to saturate the amylose component than did the dispersion whose titration was started 25 minutes after the addition of the potassium iodide. The difference should thus roughly correspond with the difference in the quantities of iodine liberated from potassium iodide by similar amounts of ellagic acid acting, respectively, for 15 and for 25 minutes. Calculations based on the titration data of the "25-minute" sample thus indicated a lower "apparent amylose content" than that of the "15-minute" sample. These values may be compared with the true amylose content of elm starch, namely 20·5%, which was estimated after preliminary 'removal' of the liberated iodine by the procedure outlined above. The graph for this titration is given in Fig.XXIII.

Potentiometric iodine titration of oak sapwood starch was previously reported to indicate the absence of an amylose component in this starch (187). This result was due, no doubt, to the complete saturation of the amylose component of the oak starch sample by iodine liberated from the potassium iodide prior to the commencement of the actual titration, the graph for which thus failed to yield a point of inflection typical of an amylose-containing starch.

The amylose contents estimated for the various

I Potentiometric Iodine Titration of 20 mgs. Elm Sapwood Starch
Titration started 15 mins. after addition of KI

II Potentiometric Iodine Titration of 19·58 mgs. Elm Sapwood Starch
Titration started 25 mins. after addition of KI

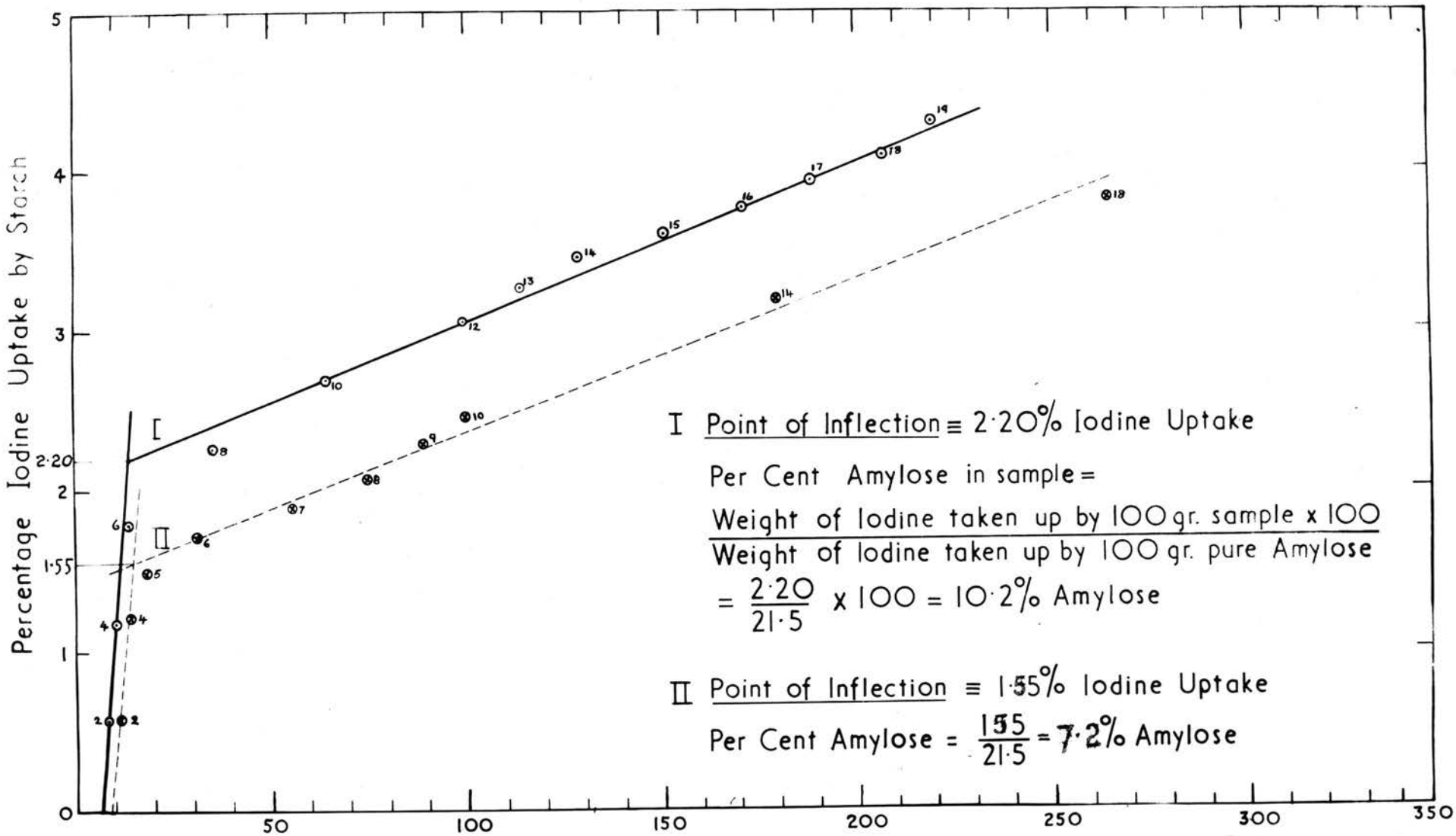



FIG. XXII.

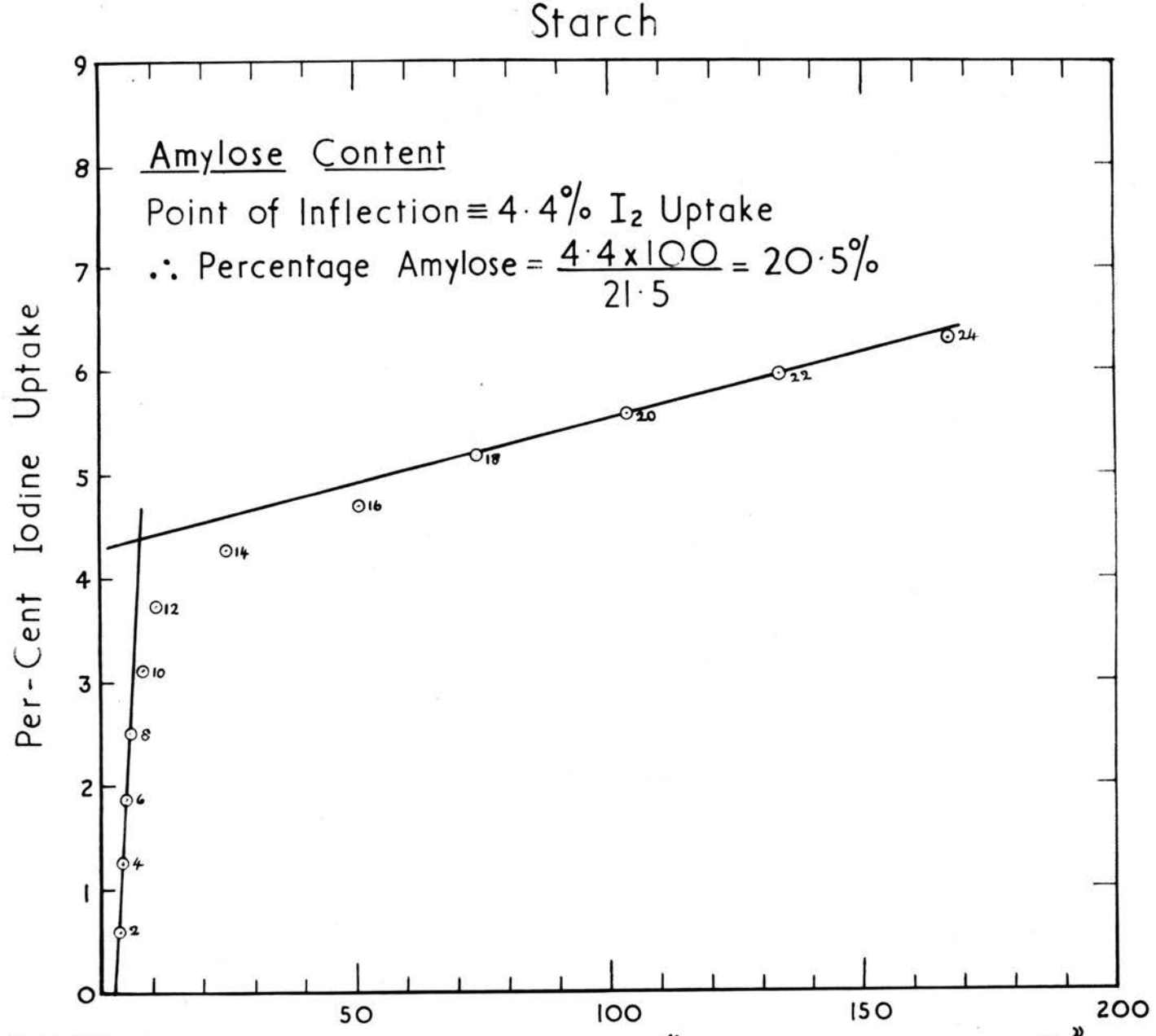

Starch
Amylose Content
Point of Inflection ≡ 4·4% $I_2$ Uptake
∴ Percentage Amylose = $\frac{4\cdot4 \times 100}{21\cdot5}$ = 20·5%
Per-Cent Iodine Uptake
0
1
2
3
4
5
6
7
8
9
50
100
150
200
2
4
6
8
10
12
14
16
18
20
22
24
Free Iodine Concentration in "mols per litre x 2 x $10^7$"

FIG. XXIII.

wood starches by the modified procedure outlined above are given in Table IV, column A. The presence of an amylose component in elm and oak sapwood starches was confirmed by its isolation in each case in typical spherocrystalline form (87) by the fractionation procedure recommended by Higginbotham and Morrison (102).

| Source. | A. Amylose Content. Pot.Iod.Titn. | B. Calc.from B.V. in brackets. |
|---|---|---|
| Elm sapwood | 20·5% | 22·2% (0·337). |
| Maple " | 18·9% | 9·7% (0·176). |
| Turkey oak " | 17·4% | 18·0% (0·283). |
| Oak " | 13·9% | 19·2% (0·306). |
| Oak " (degraded sample), | 12·3% | 8·5% (0·161). |

TABLE IV.

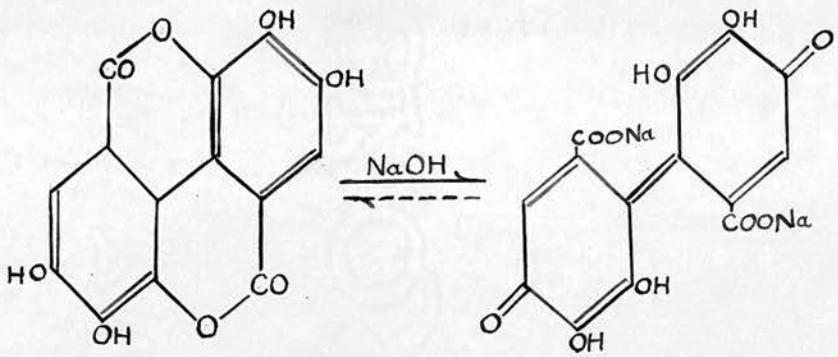
The suggestion that ellagic acid (arising from atmospheric oxidation of gallotannin in alkaline medium) was fundamentally responsible for the oxidation of potassium iodide to free iodine during the preparation of wood starch dispersions for amylose estimations is made on the basis of the following experiments and observations. An authentic sample of gallotannin was oxidised according to the directions of Nierenstein and co-workers (211) by dissolving it in sodium bicarbonate solution (0·5N) and bubbling air continuously through the resulting solution for a

period of several hours. The precipitate which was thus produced consisted of the sodium salt of ellagic acid. It was isolated and treated with dilute mineral acid to yield free ellagic acid which was recrystallised from pyridine solution. The glucose-containing fraction of the gallotannin which resulted simultaneously from this oxidation was isolated from the alkaline solution (211). In the absence of any detailed knowledge of the structure of this residue, Nierenstein designated it as "Fraction B."

Portions of ellagic acid and of "Fraction B" were dissolved separately in potassium hydroxide (0·5N) which, after 30 minutes, was neutralised with hydrochloric acid (0·5N). A few drops of starch paste were added to each solution followed by potassium iodide solution (0·5N). After about 20 minutes the solution containing ellagic acid was coloured purple while that containing "Fraction B" remained uncoloured. The former solution was rendered colourless by the addition of a few ml. of dilute sodium thiosulphate solution. It seems very likely, therefore, that ellagic acid was responsible for the effect observed under similar conditions with the wood starch dispersions.

A purely speculative explanation of the behaviour of ellagic acid towards potassium iodide is its

possible conversion, wholly or partially, to a quinonoid type of structure during the preliminary treatment with alkali. Such a conversion which resembles the change undergone by phenolphthalein in alkaline solution is represented by Fig.XXIV.




ELLAGIC ACID.

FIG. XXIV.

In the absence of supporting experimental evidence, however, it can only be said that a quinonoid structure of this type could be expected to behave in a manner similar to that actually observed since it could conceivably possess sufficiently powerful oxidising activity to cause the liberation of iodine from potassium iodide.

It may be noted, incidentally, that complete decomposition of ellagic acid to products which do not exhibit any tendency to oxidise potassium iodide may be brought about by dissolving it in potassium hydroxide (0·5N) and exposing the solution to the prolonged action of atmospheric oxygen. This means that the formation of ellagic acid by atmospheric

oxidation of alkaline solutions of gallotannin is but transitory, the ellagic acid itself undergoing further oxidation by continued treatment with the same reagents. It appears that in the continued oxidation of ellagic acid, the pH of the medium is the critical factor. In this connection, Nierenstein (211) observed that the highest yields of ellagic acid were obtained from gallotannin by oxidation in weakly alkaline media, sodium bicarbonate (0·5N) giving the best results. The utilisation of these facts in the preparation of wood starches for oxidation with potassium meta-periodate is described later.

It may also be noted that not only gallotannin, but gallic acid and various samples of lignin, namely, beech lignin, commercial "alkali" lignin, Braun's spruce "native" lignin as well as a sample of lignin isolated from elm starch granules, all exhibited similar oxidising activity toward potassium iodide after treatment with potassium hydroxide (0·5N) for 2-3 hours followed by neutralisation with hydrochloric acid (0·5N). The effectiveness of the lignins in this regard, however, was found to be much weaker than that of gallotannin under the same conditions, and it is considered possible that the lignin samples contained small quantities of the latter as an impurity.

The blue-values (94,100) of the wood starches were determined in an attempt to confirm the amylose

contents estimated by potentiometric iodine titration. In the absence of any figures for the blue-values of the pure amyloses and amylopectins of wood starches, calculation of the amylose contents from the blue-values of the 'whole' starches was based upon the arbitrarily chosen blue-values of 1·35 for amylose and of 0·05 for amylopectin (212). For comparison with the results of the potentiometric iodine titrations, the amylose contents thus calculated from the blue-values are incorporated, along with the respective blue-values, in Table IV, column B. Inspection of this table reveals very poor agreement between the two sets of results for three of the starches and only moderate agreement for the other two. Even though the amylose contents calculated from blue-values were based upon arbitrary "standard" values for amylose and amylopectin, the agreements, particularly in the case of maple sapwood starch, should be much better than they are. It is difficult to explain the widely divergent values in this case since the results of gallotannin (or ellagic acid) activity upon potassium iodide present in the solution should be negligible compared with the relatively large quantity of iodine added to develop the blue colour. The inherent brown colour of wood starch dispersions, likewise, would not be expected to upset blue-value determinations because the great degree

of dilution of the samples (viz. 5 mg. per 500 mls. soln.) rendered this brown colour almost imperceptible.

Attempts to determine the size of the repeating-units of the amylopectin components of wood starches by titration of the formic acid liberated on periodate oxidation (178, 163,180) of the samples were also seriously upset by the reactivity of the impurities. It was found that the quantity of acidic material (calculated as formic acid) which was generated by oxidation of elm starch with periodate for 150 hours corresponded with a repeating-unit for the amylopectin component of only 7 glucose units. The results of periodate titration of maple starch likewise indicated a short amylopectin repeating-unit of 6-7 glucose units and similar results were recorded by Halsall (187) for oak and turkey oak sapwood starches. The values thus suggested by periodate oxidation data have been shown to be erroneous, at least in two cases, by the results of the more reliable technique of end-group assay performed on the methylated starches. Using this technique, F. Brown (213) estimated the occurrence of one non-reducing terminal group per 18 glucose residues in methylated oak sapwood amylopectin. In the course of the present investigation an end-group assay has been performed on methylated elm sapwood starch, the amylopectin component of which has been found to possess a unit-chain of 20 glucose residues.

On the basis of repeating-units of this order of magnitude, the yields of formic acid which theoretically should be liberated from these starches on periodate oxidation should be of the order of one half to one third of the quantity of acidic material actually obtained in practice. An attempt to explain the observed excessive production of acid was made by Halsall (187) who proposed that wood starches may possess an unusual structural feature or features. The possibilities suggested for oak sapwood amylopectin were (a) the presence of sugar residues linked only through $C_1$ and $C_6$, (b) the presence of uronic acid end-groups, and (c) that the amylopectin possessed a very low mol. wt., the excess acidity arising from the reducing end-groups. On attack by the periodate ion, each of the structures (a) and (b) incorporated in an amylopectin molecule would also yield formic acid in excess of that liberated from the non-reducing terminal residues. At least in the case of elm sapwood starch, none of these explanations appears to be applicable in that, (a) paper chromatography revealed the presence of only one trimethyl glucose (viz. 2:3:6) among the hydrolysis products of the methylated polysaccharide, whereas the presence of glucose residues linked solely through $C_1$ and $C_6$ should be manifest by the detection also of 2:3:4 trimethyl glucose; (b) the absence of uronic acid residues was

strongly suggested by the results of some work described later, and (c) the viscosity of the methylated starch in m-cresol indicated a mol.wt. of nearly half a million. It seemed quite probable, therefore, that the excess acid was not derived from the actual polysaccharide material of the wood starch granules but that it arose incidentally as the result of an attack by the periodate ion upon the associated impurities.

Experiments were, therefore, conducted to ascertain how the authentic sample of gallotannin and the various lignin samples behaved when treated separately with the periodate ion under conditions identical with those employed for the periodate oxidation of polysaccharides. It was found that the lignins (including a sample isolated from elm starch) yielded negligible quantities of acidic material by comparison with the quantity produced from gallotannin. In a typical experiment, a solution of gallotannin (0·0268 g. in 60 ml. water), neutralised by the addition of dilute alkali, was shaken with an excess of solid potassium periodate. A red-brown flocculent precipitate first formed which slowly dissolved to give a lemon-yellow solution which no longer gave the deep blue coloration characteristic of the original gallotannin solution upon the addition of a little ferric sulphate. After 160 hours the solution was

titrated with dilute alkali using methyl red as indicator, and the titre found to correspond to about 21 ml. 0·01N NaOH. Assuming that acidic material was liberated to a similar degree from the gallotannin impurity of the wood starch samples and that this impurity occurred in the samples to the extent of some 4-5%, an adequate explanation could readily be made of the yield of acidic material produced in excess of the formic acid theoretically expected on periodate oxidation of a normal amylopectin structure possessing a repeating-unit of 18-20 glucose residues.

It was not considered possible, however, to estimate accurately the size of the amylopectin repeating-units by making compensatory calculations for the effect of the gallotannin impurity upon the results of periodate oxidations. Such calculations were rendered impracticable, in the first place, by the difficulty of accurately determining the gallotannin contents of the various wood starches and, secondly, by the possibility that these gallotannins may not be identical one with another and with the "standard" sample used in the above test. They may, in other words, each differ to the extent to which they liberate acidic material under identical conditions of periodate oxidation. Attempts were, therefore, made to remove the impurities from elm and oak sapwood starches and thus obtain samples sufficiently

pure to eliminate complicated side-reactions during periodate analyses. The attempted purifications are outlined below along with the results of periodate titration of the recovered samples.

1) Attempted Solvent Extraction of the Impurities. Elm starch granules were suspended in the following solvents, hot and cold: alcohol, dioxan, pyridine, acetone, chloroform, benzene and light petroleum. No obvious colour was imparted to any of the solvents and subsequent evaporation to dryness of the filtered solvents yielded either mere traces of a residue or none at all. The granules still retained their original colour.

2) Attempted Purification by Solution in Perchloric Acid. Perchloric acid effects rapid solution of starch at room temperatures, and it is said that 4·8 normal aqueous solutions of the acid represent an optimum concentration which brings about least degradation of the dissolved polysaccharide (214). This claim was re-investigated in the present work and periodate titrations were performed on potato and "defatted" waxy maize starches both before and after solution in perchloric acid (4·8N) for 30 minutes. The values thus calculated for the proportions of non-reducing terminal residues in the starches were found to be identical within the limits of experimental

error with the respective values obtained for the untreated starches (Table V). But mol.wt. determinations by colorimetric estimation of the products of reduction of 3:5 dinitrosalicylic acid according to the directions of Meyer (166) indicated lower apparent mol.wts. for the perchloric acid-treated starches than those indicated by the same method for the original granules. In view of the relatively low values recorded for the latter (Table V), it was obvious that Meyer's technique does not provide absolute mol.wts., particularly when the values approach the extremely high order of undegraded amylopectin molecules. It has been found useful, however, as a quick and convenient method for obtaining relative data, and it seems significant that, in each case, the acid-treated starches gave the lower values. It was concluded, therefore, that treatment with perchloric acid (4·8N) under the conditions described in the experimental section does, in fact, bring about some degradation which appears not to be sufficiently extensive to affect materially the results of periodate end-group analyses.

Properties of Starches Before & After Soln. in Perchloric Acid.

| Analyses performed on starches:- | Potato. | Waxy Maize. |
|---|---|---|
| (1) Before Treatment. | | |
| Amylopectin repeating-unit | 24 | 22 |
| Mol.Wt. | $2{\cdot}05 \times 10^5$ | $2{\cdot}52 \times 10^5$ |
| (2) After Treatment. | | |
| Amylopectin repeating-unit | 23 | 20-21 |
| Mol.Wt. | $9 \times 10^4$ | $1{\cdot}31 \times 10^5$ |

TABLE V.

By analogy, it therefore seemed reasonable to assume that preliminary treatment with 4·8N perchloric acid would not bring about serious degradation when applied also to wood starches. Solution of elm starch in perchloric acid (4·8N) occurred readily, leaving the impurities in the form of a fine, dark-brown suspension which was removed by centrifuging and shown to constitute some 7% by weight of the starch sample dissolved. The clear centrifugate was neutralised by the addition of sodium hydroxide solution, dialysed for a short time and the starch then recovered by precipitation into absolute alcohol. The dried sample was obtained as a fluffy, cream-coloured powder, B.V. 0·34. Estimation of the glucose formed on hydrolysis indicated that the sample was only 89% pure. It was also shown to retain some gallotannin activity and it was not surprising, therefore,

that the results of periodate oxidation indicated for the amylopectin component an apparent unit-chain of 14 glucose residues.

Recovery of the sample dissolved in perchloric acid was also attempted by neutralisation followed by precipitation of the starch as the starch-iodine complex (214). This was decomposed by means of alcoholic sodium hydroxide and the isolated sample, on oxidation with periodate, yielded a similar quantity of acidic material to that yielded by the previous sample.

3) Purification (with accompanying degradation) by Treatment with 0·5% Ethanolic-hydrogen chloride. Treatment of granular oak sapwood starch with this reagent under the conditions described by Baird, Haworth and Hirst (215) yielded a product very similar to that obtained by these workers from potato starch granules. The recovered oak starch retained its granular form but microscopic examination revealed that the great majority of the granules had been damaged to varying extents. Longitudinal indentations could be observed on the surfaces of most granules and a few seemed to be cleanly and almost completely severed. The recovered sample possessed the same buff colour as the original, but it was found that the treated granules dispersed readily in hot water leaving the impurities suspended in very finely divided state.

Removal of this dark-brown suspension by filtration left a faintly opalescent, mobile, colourless solution of the starch, the tendency to paste-formation of which had been destroyed. The filtrate was poured into alcohol and the starch was thus precipitated as a pure-white solid which possessed very slight reducing action towards Fehling's solution and gave a deep blue colour with iodine, B.V. 0·23 corresponding with an amylose content of 13·9%. Potentiometric iodine titration of the sample indicated the presence of 14·2% amylose. These values were then in satisfactory agreement with the value of 13·9% amylose determined by potentiometric iodine titration of the original oak starch sample (Table IV). A significant difference between the potentiometric titrations of oak starch before and after treatment with ethanolic-hydrogen chloride was the considerably greater E.M.F. at which the treated oak amylose adsorbed iodine during the titration. It was observed by Whistler and co-workers (216) that the lower the mol.wt. of an amylose, the higher the potential at which it takes up iodine during potentiometric iodine titration. These facts suggest that the oak amylose had been degraded to shorter chains by treatment with ethanolic-hydrogen chloride which, it therefore appears, is capable of random hydrolysis of chains of 1:4 links. It follows, then, that the repeating-units of the

amylopectin molecules would also be liable to attack. This deduction was confirmed by the results of periodate titration of the recovered oak starch and also of a sample of potato starch treated under identical conditions with 0·5% ethanolic-hydrogen chloride. That extensive degradation had occurred in each of the recovered samples was indicated by colorimetric mol.wt. determinations by the 3:5 dinitrosalicylic acid method of Meyer (166). The mol.wt. of the recovered oak starch was found to be of the order of $2{\cdot}6 \times 10^4$ (i.e. D.P. 160 anhydroglucose units) while that of the recovered potato starch was found to be considerably lower, namely, $1{\cdot}45 \times 10^4$ (i.e. D.P. 90). This latter value is roughly comparable with those determined viscometrically for acetates of the similarly modified potato starch of Baird, Haworth and Hirst (215) who recorded D's.P. of 73 and of 120 for two of their products.

On the assumption that the amylose components of the recovered starches had been degraded to some extent and that the extra end-groups thus liberated may have contributed appreciably to the formic acid yields estimated by periodate titration, the results of the latter were calculated in terms of "average number of glucose residues per non-reducing terminal residue" for the 'whole-starch' samples. Such functions could then be compared with similar ones

calculated for the original samples, and the extent of hydrolytic scission of 1:4 linkages during purification thus assessed. In making the calculations in the cases of the low mol.wt. recovered starches it was necessary to correct for the contribution of the reducing end-groups to the total yield of formic acid liberated by oxidation with periodate. For example, recovered oak starch (0·2938 g.) yielded a quantity of formic acid equivalent to 12·53 ml. 0·01N sodium hydroxide during periodate oxidation for 150 hours. The results of the mol.wt. determination recorded above indicated the occurrence of 1 reducing end-group per 160 glucose residues, that is, per $2·6 \times 10^4$ g. of modified oak starch. Since, according to Fig.XI, one reducing end-group is capable of liberating 2 moles of formic acid on periodate oxidation, $2·6 \times 10^4$ g. of modified oak starch would have given rise to 2 moles of formic acid <u>in excess</u> of that liberated from the non-reducing terminal residues. If, then, the reducing end-groups in $2·6 \times 10^4$ g. oak starch liberated formic acid $\equiv$ 2000 ml. N.NaOH, $\therefore$ those in 0·2938 g. oak starch liberated formic acid $\equiv$ 2·26 ml. 0·01N.NaOH. $\therefore$ the <u>non</u>-reducing end-groups in 0·2938 g. oak starch liberated formic acid $\equiv$ 12·53-2·26 = 10·27 ml. 0·01N.NaOH. Now, if 10·27 ml. 0·01N.NaOH were required to neutralise the formic acid liberated from non-reducing end-groups of

0·2938 g. oak starch, 1000 ml. 0·01N.NaOH would be required for that from 2,860 g. oak starch, that is, one mole of formic acid would be liberated from 2860 g. oak starch. Since one mole of formic acid was expected per non-reducing end-group, the"average number of glucose residues per non-reducing terminal residue,"

$$= \frac{2860}{162} = 17{\cdot}6$$

The corresponding value calculated for the original oak starch on the bases of a 14% content of amylose and an amylopectin repeating-unit of 18 glucose residues (213), is approximately 21. The absence of gallotannin in the treated sample was fairly definitely established and its aqueous solution prior to periodate oxidation was shown to be quite neutral to methyl red. It, therefore, appears that the difference between the above values was indeed due to the exposure of an increased proportion of non-reducing end-groups during treatment with the ethanolic-hydrogen chloride. This can only mean that hydrolytic scission of a certain proportion of 1:4-linkages had occurred in addition to the very probable scission of some of the amylopectin inter-unit bonds indicated by the relatively low D.P. of the sample.

These conclusions are confirmed by comparison of the values for "average number of glucose residues per non-reducing terminal residue" calculated from

periodate oxidation data for 'whole' potato starch before and after treatment with the acid reagent. The values in this case are, respectively, 29 and 20 to the nearest whole numbers.

It is difficult to reconcile these results with that obtained by Baird, Haworth and Hirst (215) for the end-group assay performed on the methylated derivative of their similarly modified potato starch. Treatment with ethanolic-hydrogen chloride appeared to have brought about complete disaggregation of the amylopectin molecules into single repeating-units. The results calculated from the methylation data precluded the possibility of extensive hydrolytic scission of 1:4 links in that the proportion of "end-group" obtained was almost identical with that normally observed for ordinary samples of potato starch.

In view of the great reduction in mol.wt. brought about by this treatment, however, it was not further considered as a satisfactory method of purification of the wood starches although it did effect more or less complete removal of the associated impurities.

It is of interest in this connection to recall the method used by Link and co-workers (217) for the extraction of starch from the wood of the apple tree. The starch-containing wood meal was first refluxed for 30 minutes with 85% ethanol containing 1% nitric acid. It was reported that although this treatment did not

destroy the granular structure of the starch, subsequent heating in 20% ethanol effected its dispersion without previous swelling of the granules. The purified sample was isolated as an amorphous white powder soluble in warm water. Mr. W.G. Campbell kindly supplied us with samples of oak and apple sapwood starches which had been isolated in this manner and they were oxidised with periodate in the hope that contamination with interfering impurities had been avoided by the method of isolation. The values for "average number of glucose residues per non-reducing terminal group" for the 'whole' starches were calculated as 10-11 and 14-15 for these samples of oak and apple starches, respectively. Obviously, the corresponding values for the amylopectin components of the respective starches would be even lower than these values. Estimations of the amylose contents of the samples for the accurate calculation of the apparent lengths of these amylopectin repeating-units was, therefore, considered not worth-while, since enough could be gathered from the above results to indicate that either periodate-reactive impurities were still present or that the samples had been dextrinised during their extraction.

4) Attempted Purification by Dispersion in Sodium Hydroxide Solution. In view of the varying degrees of degradation of starches brought about by the above acidic reagents, it was decided to examine the effect of dispersion of the crude wood starches in an alkaline medium. In the initial experiments, the possibility of oxidative degradation of such suspensions by atmospheric oxygen was reduced by conducting them in an atmosphere of nitrogen. A dispersion of elm starch (2%) in sodium hydroxide solution (2N) was accordingly prepared in a sealed bottle in which the air had been replaced by nitrogen. Suspended dark-brown particles of impurities were removed by centrifuging and the clear, brown supernatant liquid neutralised by the addition of acetic acid. The solution was dialysed for a short time and the starch then precipitated into alcohol. The starch was recovered as a fluffy, cream-coloured powder which dispersed readily in warm water and gave a deep blue colour with a little iodine solution, B.V. 0·311 corresponding with an amylose content of 20·2%, a value in excellent agreement with that obtained by potentiometric iodine titration of the original sample (Table IV). The actual starch content of the dried sample was shown to be approximately 94% by quantitative estimation of the glucose formed on hydrolysis with dilute sulphuric acid.

The yield of acidic material (calculated as formic acid) which was obtained after periodate oxidation of the sample for 150 hours corresponded with an amylopectin repeating-unit of 10 glucose units only. It was considered unlikely that this method of attempted purification of the starch had brought about degradation to the extent suggested by this figure and it was concluded that the 'purification' was only partially successful.

The complete removal of interfering impurities from the wood starches (without accompanying degradation of the starches) thus seemed to be very difficult, if not impossible, and it was therefore decided to attempt a compromise. It was found that complete disruption of the gallotannin impurity into products which displayed little or no obvious reactivity toward the periodate ion could be accomplished by prolonged atmospheric oxidation in alkaline medium. The technique finally adopted was developed on the basis of the following experimental results and observations. Ellagic acid and Nierenstein's "Fraction II" (211), formed by atmospheric oxidation of gallotannin dissolved in sodium bicarbonate solution, were separately oxidised with periodate for 150 hours. It was found that while ellagic acid was attacked under these conditions to yield appreciable quantities of acidic material, the periodate solution containing

"Fraction II" remained practically neutral. As mentioned previously, ellagic acid in more strongly alkaline media than 0·5N sodium bicarbonate solution is destroyed by prolonged contact with atmospheric oxygen. It was reasoned, therefore, that prolonged contact with atmospheric oxygen of the gallotannin impurity adsorbed on the wood starches dispersed in sodium hydroxide solution should effect the removal (by destruction) of the periodate-reactive ellagic acid formed in the initial stages of atmospheric oxidation, probably leaving the unreactive "Fraction II" adsorbed on the starches. The experimental conditions under which this effect was achieved were, briefly, as follows. Air was vigorously bubbled as a continuous stream for 20 hours through 1% dispersions of the various wood starches in sodium hydroxide solution (0·5N). In each case, an insoluble olive-green residue was thus obtained which displayed none of the properties characteristic of ellagic acid. The starches were recovered by neutralisation of the clear alkaline dispersions followed by dialysis for 2-3 weeks, and then precipitation of the polysaccharides into alcohol. Even after such relatively long periods of dialysis each of the recovered starches retained small but measurable quantities of alkali. Before treatment with periodate it was, therefore, essential to neutralise the aqueous suspensions of the

samples to be analysed by the addition of hydrochloric acid (0·01N). Difficulty was experienced in the colorimetric detection of the end-points of these neutralisations due to the varying depths of coloration of the suspensions. The colour change of methyl red could be detected by matching against controls in the cases of elm, oak and turkey oak starches. In the case of maple starch, however, the change from yellow to pink of methyl red could not be seen against the brownish background. Phenolphthalein was employed for the detection of this end-point but, again, a control suspension was necessary against which to match the "neutral colour." This phenolphthalein titre was "corrected" to pH 7 on the assumption that the alkali in the starch samples consisted of sodium carbonate to which, it was reasoned, any free sodium hydroxide originally present would be converted on exposure to the atmosphere during dialysis. Unfortunately, detection of these end-points was not possible by potentiometric titration using an antimony electrode against a saturated calomel cell. The curve obtained by plotting "measured E.M.F." against the "volume of 0·01N hydrochloric acid added" to an aqueous suspension of the recovered maple starch did not slope sharply, in characteristic fashion, at the end-point. Instead, the curve sloped gently and smoothly through pH 7. A possible explanation of

this behaviour was a buffering effect due to the presence of phenolic bodies which could conceivably have been formed during the atmospheric oxidation of the gallotannin impurity and which remained adsorbed on the starch. The neutralised starch suspensions were oxidised with periodate and from the results of subsequent titrations it was estimated that more or less normal quantities of acid (calculated as formic acid) were liberated after 150 hours treatment. The chain-lengths thus calculated for the repeating-units of the various wood-starch amylopectins are as follows: elm amylopectin 17-18, oak 18, turkey oak 17-18, and maple 16 glucose units.

A dispersion of potato starch (1%) in sodium hydroxide (0·5N) was "atmospherically oxidised" in a manner identical with that outlined above for the destruction of gallotannin adsorbed on the wood starches. The recovered potato starch was oxidised with periodate and the quantity of formic acid liberated after 150 hours found to correspond to a repeating-unit for the amylopectin component of 24 glucose residues, a value identical with that obtained for untreated potato starch. This indicates that prolonged treatment of alkaline dispersions of starches with atmospheric oxygen does not bring about such undue oxidative degradation of the starch macromolecules as to affect the results of periodate titration to any

measurable extent. It was, therefore, considered reasonable to infer that the results recorded above for the wood amylopectins are quite valid and that the values do at least approach the true sizes of the repeating-units of the undegraded amylopectins.

Attempted Estimation of the Relative Proportion of Inter-unit Bonds of the 1:6 Variety to the Possible 1:3 and 1:2 Varieties in Elm Sapwood Amylopectin.

A known weight of the original elm starch granules was shaken as an aqueous suspension with excess of solid potassium metaperiodate for 14 days and the oxidised polysaccharide, still in granular form, was isolated and hydrolysed as recommended by Halsall, Hirst, Jones and Roudier (27,185,187). An appropriate quantity of D-ribose was weighed into the acid hydrolysate which was then neutralised and worked up as recommended by the above workers. The neutral solution thus obtained was evaporated to dryness and a portion of the residual sugars (dissolved in a little water) transferred to the starting-line of a quantitative paper chromatogram. The sugars were then separated according to the general instructions of Partridge (218). The longitudinal marginal strips were removed from the dried paper, sprayed with ammoniacal silver nitrate solution and 'developed' by heating in an oven at 105°. Three spots appeared after 10-15 minutes,

two of these corresponding with glucose and D-ribose, and the third, a dark-brown spot with an $R_G$ value of 0·303 was probably due to D-erythrose (219). Quantitative estimations of the glucose and the ribose were accomplished by the method described by Flood, Hirst and Jones (186). The results of two such quantitative chromatograms were interpreted on the assumption that all the glucose residues of the amylose component of the elm starch were attacked during periodate oxidation and that, therefore, none of the glucose recovered after hydrolysis of the oxidised 'whole' starch arose from this component. It was assumed, in other words, that this recovered glucose was liberated by hydrolysis of the periodate-oxidised amylopectin component exclusively. Calculations made on this basis indicated that the amylopectin component yielded 3·6% of its own weight of glucose. Now, an end-group analysis performed on methylated elm starch revealed that the amylopectin repeating-units consist of some 20 glucose residues. Hence, the total number of trisubstituted glucose residues (represented by Figs. XIII, XIV and XV) which are involved in inter-unit bonding within the amylopectin molecule would, when calculated as free glucose molecules, represent 5·55% by weight of the total amylopectin. Since only those glucose residues which take part in inter-unit bonding through $C_2$ and/or $C_3$

are theoretically recoverable as glucose on hydrolysis of the periodate-oxidised amylopectin, it appears that such residues constitute approximately 65% of all glucose residues taking part in inter-unit bonding. It follows then that 35% at least of these glucose residues are involved in inter-unit bonding through $C_6$. That is, it appears that the majority of inter-unit bonds are other than the 1:6 variety and this would mean that at least in respect of inter-unit bonding, elm amylopectin is unique. However, the unusually high yield of glucose, which was obtained on hydrolysis of the periodate-oxidised starch and which was responsible for the apparently anomalous results recorded above, can be explained reasonably on the basis of the associated gallotannin impurity. According to Fischer (220), the structure of gallotannin is that of a penta-acylated glucose as shown in Fig. XXV, the particular acyl group being the m-digalloyl residue.

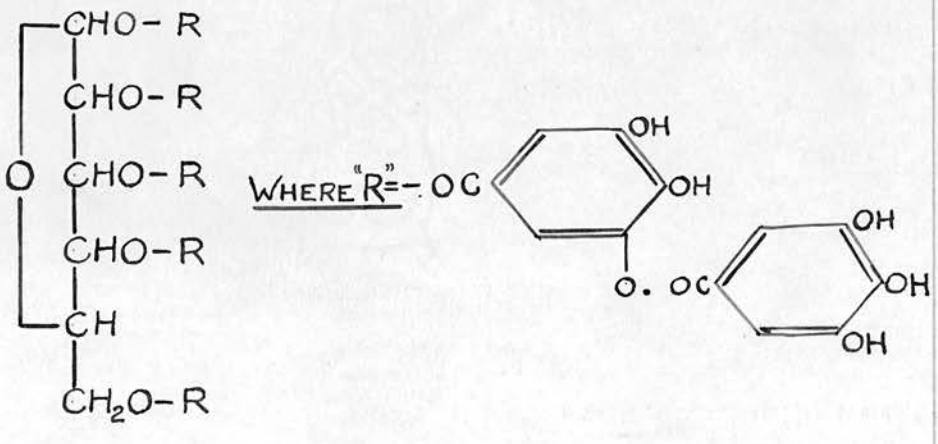



FIG. XXV. Gallotannin (according to Fischer).

All the hydroxyl groups of the glucose residue being thus substituted, it should be protected from attack by the periodate ion. An attempt to prove this point has been made in the present work and an aqueous solution of authentic gallotannin was shaken with excess of solid potassium metaperiodate for ten days. Unfortunately, it was not found possible to conduct the experiment on a quantitative basis owing to the difficulty of effecting a clear-cut separation of the inorganic salts from the residual periodate-oxidised gallotannin fraction. The latter (only partially separated from iodine-containing salts) was hydrolysed by treatment with dilute sulphuric acid for 10 hours. The hydrolysate was neutralised and freed as far as possible from inorganic ions, and then evaporated to dryness. A portion of the residual solid, dissolved in a little water, was transferred to the starting-lines of chromatogram papers which were then irrigated with butanol saturated with water under the conditions prescribed by Partridge (218). Development of the papers, one with ammoniacal silver nitrate and the other with aniline oxalate, revealed the presence in each case of small but definite spots corresponding in colour and position with those of the glucose controls. Assuming the gallotannin impurity of the elm starch to be constituted similarly to the authentic sample and that the periodate-oxidised fraction remained strongly

adsorbed on the periodate-oxidised polysaccharide molecules during the subsequent working-up of the granules, it would follow that hydrolysis of the latter would simultaneously liberate "excess" glucose from the gallotannin residue. A rough estimate of this excess glucose can be obtained assuming the presence of 5% of gallotannin impurity calculated on the weight of the original elm starch granules. This would mean that some 15 mg. of gallotannin would be subjected to the activity of the periodate ion along with the elm starch in the above experiment and would liberate its contained glucose on subsequent hydrolysis. On the basis of Fischer's formula, 15 mg. gallotannin should liberate 1·6 mg. glucose on hydrolysis. Since the total yield of recovered glucose was estimated as 6·73 mg., it follows that the periodate-oxidised amylopectin component was responsible for the liberation of about 5·1 mg. glucose which represents some 2·7% by weight of the original amylopectin. Following the lines of the previous calculation it may be estimated that some 51% of the inter-unit bonds of elm amylopectin are represented by the 1:6 variety. Whether or not the above compensatory calculations are wholly justified remains to be decided by the results of further experimental work. It may be purely fortuitous that the value of 51% for the proportion of 1:6 to other inter-unit bonds in elm

amylopectin now agrees closely with the corresponding value of about 50% calculated for both barley (182) and acorn amylopectins (27) from data for the 'whole' starches.

It may be observed, incidentally, that the formula proposed for gallotannin by Nierenstein (221) is not consistent with the above-described isolation of glucose by hydrolysis of periodate-oxidised gallotannin. Nierenstein's impression of gallotannin as a glucoside of polydigalloyl-leucodigallic acid anhydride is reproduced in Fig.XXVI.

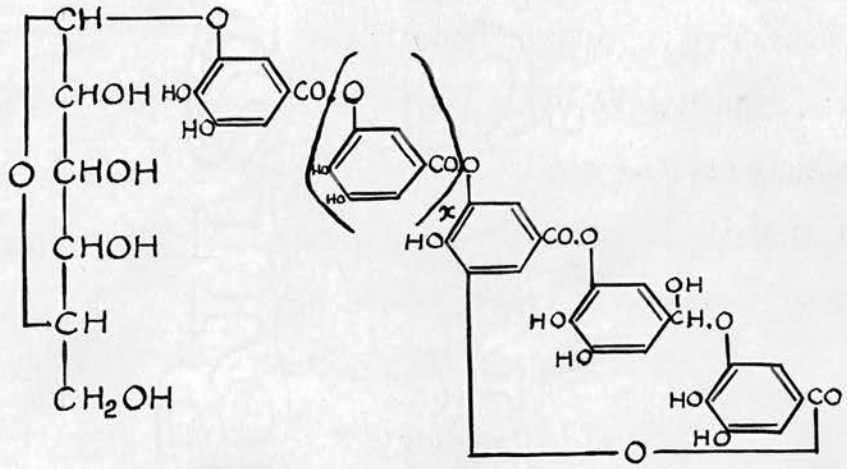


FIG. XXVI. Gallotannin (according to Nierenstein).

In such a molecule the hydroxyl groups attached to $C_2$, $C_3$, $C_4$ and $C_6$ of the glucose residue would be free and the latter would thus be disposed to attack by the periodate ion. No glucose would then be yielded by subsequent hydrolysis of the recovered gallotannin residue.

Analyses of the Actual Starch Contents of the Wood Starch Samples. This determination was performed on each of the five wood starch samples in the usual manner, that is, by quantitative estimation of the glucose formed on acid hydrolysis of an accurately weighed sample of each starch. In each case, insoluble brown residues remained after hydrolysis with normal sulphuric acid at 100° for 7 hours. This flocculent residue, in the case of elm starch, did not appear to undergo any change on continued heating with the dilute sulphuric acid for a further 10 hours. The residue from each hydrolysis was collected in a Gooch crucible, dried and weighed, and the results expressed as a percentage by weight of the starch sample hydrolysed. The results are listed in the final column of Table VI. These insoluble impurities displayed positive reactions to the so-called "Maüle test" (223) for lignin (described in the experimental section).

The glucose liberated by hydrolysis of the various samples was estimated using Somogyi's copper reagent (222), and in the cases of turkey oak and the two samples of oak starch, confirmation of the analyses was attempted by means of hypoiodite oxidation. The results are incorporated in Table VI for comparison.

| Source of Starch. | Moisture content of air-dried granules. | Starch content determined by hydrolysis and estimation of liberated glucose by:- (a) Somogyi reagent | (b) hypoiodite. | Insoluble Residue. |
|---|---|---|---|---|
| Elm sapwood | 12·20% | 78·5% | - | 4·6% |
| Maple " | 14·76% | 75·5% | - | - |
| Turkey Oak sapwood | 14·00% | 76·1% | 74·0% | 8·1% |
| Oak " | 13·42% | 78·8% | 73,2% | 5·8% |
| Oak " (degraded) | 14·10% | 75·0% | 73·0% | 5·6% |

TABLE VI.

It is perhaps significant that analyses of the liberated glucose by hypoiodite oxidation (in the latter three cases) yielded results which are lower than the corresponding results for which the glucose was estimated by means of the Somogyi technique. The discrepancy is particularly obvious in the case of the undegraded oak starch. It is thought that the discrepancies were primarily due to the liberation of gallic acid from gallotannin during sulphuric acid hydrolysis of the starch samples. It was noted previously that gallic acid, like gallotannin, may be converted by atmospheric oxidation of its alkaline solution to a product which has the power to liberate iodine from potassium iodide. If such reactions

occurred in the alkaline hypoiodite medium (during oxidation of the glucose yields) and iodine was liberated from the potassium iodide which was also present in the solution, a ready explanation would thus be provided for the lack of agreement between the above two sets of results. It is quite possible, of course, that the normal mechanism of the copper reagent was also interfered with by the reactive impurities and that neither set of results is, therefore, really reliable.

For this reason, it was decided, in the case of elm starch, to separate on a quantitative paper chromatogram the glucose formed by acid hydrolysis of the sample. The separated glucose was estimated according to the directions of Flood, Hirst and Jones (186). In this manner, interfering impurities were removed, allowing a reliable estimation of the glucose to be made. The sample of elm starch was thus shown to contain 76·6% of glucose residues. This value is slightly lower than that calculated by direct estimation of glucose by means of Somogyi's reagent, namely, 78·5% (Table VI). It is difficult to decide whether the difference is significant since it probably lies within the limits of experimental error. The value of 76·6% for the actual starch content was considered the more reliable, however, and was accepted as a basis for subsequent calculations. The

analysis of elm sapwood starch is summarised in tabular form as follows:-

| | |
|---|---|
| Actual Starch Content | 76·6% |
| Insoluble Impurity (lignin?) | 4·6% |
| Moisture Content | 12·2% |
| Mineral Ash | 0·96% |
| Total | 94·36% |

Thus 94·4% of the sample can be accounted for directly, the remaining 5-6% being represented partially or wholly by the gallotannin impurity.

In 1935 Campbell (224) reported the dilute sulphuric acid hydrolysis of a sample of starch isolated from oak sapwood in amorphous (non-granular) form. This hydrolysis also yielded an insoluble dark-brown residue which constituted 6·14% of the weight of the sample. The presence in the neutralised hydrolysate of a sugar or sugars other than glucose was indicated, while distillation of the original starch with hydrochloric acid (12%) yielded a quantity of carbon dioxide which corresponded to the presence of 4·65% of uronic anhydride residues. It was, therefore, suggested that oak sapwood starch was an acid polysaccharide and that it possessed a constitution intermediate between those of starch and the wood hemicelluloses (226).

It was further reported (225) that a starch

isolated from oak leaves yielded, on distillation with hydrochloric acid (12%), a quantity of carbon dioxide corresponding to 24·4% of uronic anhydride residues. More recently, however, Campbell (234) has reported that carbon dioxide estimations performed on a sample of granular oak sapwood starch gave results of less than 0·5%. Link and co-workers (217) demonstrated the presence of a trace of xylose in their sample of apple-wood starch.

The possibility of the presence of sugar residues other than glucose has therefore been investigated in the case of elm starch. Two samples of elm starch were hydrolysed with dilute sulphuric acid (5%), one for 10 hours and the other for 1 hour. After neutralisation, each hydrolysate was spotted separately on chromatogram papers which were then irrigated for 80 hours with butanol saturated with water according to the directions of Partridge (218). Development of the dried papers with ammoniacal silver nitrate, aniline oxalate and phenetidine respectively, revealed in each case a strong spot corresponding with the glucose control and also a very faint spot corresponding with that of maltose. No other reducing sugars could be detected and the chromatograms appeared to be identical in all respects with those obtained from the hydrolysates of potato and barley starches.

The fact that the hydrolysate of elm starch

contained very little, if any, other reducing sugar than glucose is confirmed by comparison of the result of the quantitative paper chromatogram recorded above (in which only glucose was estimated) with the result of the direct estimation (Table VI) to which, besides glucose, any other reducing sugar would also have contributed. The slightly lower result from the quantitative paper chromatogram may or may not be significant but, again, this is difficult to decide because, as previously observed, the difference between the two results probably lies within the limits of experimental error.

The absence of uronic anhydride residues in elm starch was demonstrated by distillation with hydrochloric acid (12%) for 4·5 hours using the method and apparatus described by Swenson, McCready and McClay (227) for the estimation of carbon dioxide thus liberated from polyuronides. The elm starch sample was found to liberate 0·45% of carbon dioxide (corresponding with an apparent uronic anhydride content of 1·8%). Swenson and co-workers (loc.cit.) have shown that pure glucose yields about 0·3% of carbon dioxide under conditions identical with those used in the present work for elm starch. Other workers (228,26) have shown that glucose, maltose and various starches yield some 0·3 - 0·5% of carbon dioxide as determined by means of an improvement of the method

of Dickson, et.al. (229). Campbell, Hirst and Young (228) concluded that "in connection with starches, no structural significance can be attached to reported $CO_2$ yields of the order 0·3 - 0·5%, whilst for other polysaccharides, yields of $CO_2$ up to 1% may be equally unreliable as an index of the presence of uronic anhydride."

Attempts to explain the liberation of the significant quantities of carbon dioxide from the oak starch sample originally investigated by Campbell (224) included the possible decarboxylation of gallic acid (derived from the gallotannin impurity) under the conditions of the uronic anhydride determination. That this explanation was not valid was indicated by the fact that the gallotannin-contaminated elm starch did not yield significant quantities of carbon dioxide under these conditions. This point was confirmed directly by distilling authentic gallotannin with hydrochloric acid (12%) when it was found that liberation of carbon dioxide occurred to the extent of about 1% only. It was then considered that hemicellulose-containing fibrous material which was known to occur in varying proportions in the starch samples, may be a possible source of carbon dioxide in these determinations. Microscopic examination of elm starch granules revealed the presence of traces only of such fibrous material. In a "second grade" sample

of oak starch, however, the admixture of a considerable proportion of wood fibres with the granules could readily be observed. A uronic anhydride determination performed on this sample yielded 0·96% of carbon dioxide (corresponding with the presence of 3·84% uronic anhydride). This value is possibly significantly greater than that for elm starch recorded above. Further investigations upon this "second grade" oak starch sample included dilute sulphuric acid hydrolysis for 4·5 hours followed by neutralisation and concentration of the hydrolysate which was then spotted on to the starting-lines of three paper chromatograms. These were irrigated with the upper phase resulting from the separation of a well-shaken mixture of 40 mls. n-butanol, 10 mls. glacial acetic acid and 50 mls. water, according to the directions of Partridge (218). The dried chromatograms were subsequently developed with ammoniacal silver nitrate, aniline oxalate and phenetidine, respectively. Besides the appearance of a strong spot corresponding with glucose and a very faint spot for maltose, a spot of intermediate intensity was also observed which corresponded with a xylose control. No other reducing sugars, including uronic acids, could be detected. It is considered very likely that the xylose arose by hydrolysis of a hemicellulose or xylan contained in the fibrous material which contaminated this sample of oak starch.

Molecular Weight Determinations of Wood Starches.

Very low mol.wt. values have previously been recorded for these starches by the method of alkaline hypoiodite oxidation (187). It was found that 240 mg. samples absorbed some 2 mls. of iodine solution (0·05N) in alkaline medium. On the assumption that one reducing end-group occurs per molecule, mol.wts. of the order of $5 \times 10^3$ (D.P. $\simeq$ 30) were thus calculated, but it is now known that such values represent but a fraction of the true mol.wts. of the wood starches. The discrepancy is attributed, in this case, to the direct influence of the associated gallotannin impurity rather than to the reactivity of one of its degradation products. It will be recalled that interference with the results of potentiometric iodine titration of the samples consisted of an actual liberation of free iodine into the medium through the agency of ellagic acid, a product of alkaline oxidative degradation of gallotannin. In the present instance, however, the reverse effect is operative, in that excessive absorption of iodine from the medium has occurred, the mol.wts. of the polysaccharides thus being made to appear relatively very low.

Griessmayer (230) first reported that the addition of tannic acid (gallotannin) to solutions of iodine and of the starch-iodine complex caused decoloration of these solutions. He investigated the action of

iodine on gallotannin and suggested that while the iodine was reduced to hydriodic acid, the gallotannin was converted to gallic acid which was, in turn, converted to ellagic acid. Incidentally, it is of interest to note, in connection with the observation made in the present work of the strong adsorption of gallotannin on wood starches, that Griessmayer (loc. cit.) described the formation of a precipitate on the addition of gallotannin to aqueous dispersions of wheat starch. The precipitate was found to dissolve on warming and to reappear on cooling. Griessmayer applied the name "tannate of starch" to this adsorption complex.

The fact that oak sapwood contains an iodine-reactive gallotannin was demonstrated by the following qualitative experiment. The oak sapwood sawdust was extracted as a suspension (2%) with boiling water for 2-3 minutes. The suspension was then filtered to yield a clear, pale-brown solution containing wood starch and gallotannin. If dilute iodine solution was then added slowly with thorough mixing between additions, it was observed that a transient blue colour developed which quickly faded first to olive-green and then to pale brown. An appreciable quantity of iodine was necessary to impart a permanent blue colour to the solution. The presence of gallotannin in the aqueous infusion was confirmed by application

of the tests described in the experimental section.

A quantitative experiment using authentic gallotannin was performed as follows, under experimental conditions identical with those employed for hypoiodite oxidation of the starches (187). Iodine solution (10 ml.; 0·05N) was added to a solution of gallotannin (10 mg.) in water (20 ml.) buffered with sodium carbonate (0·2M)/sodium bicarbonate (0·2M) buffer (10 ml.). The mixture was allowed to stand undisturbed for one hour and the excess iodine then estimated by titration against thiosulphate solution (0·05N). The difference between this titre and that of a 'blank' experiment indicated that 9·53 ml. 0·05N iodine solution had been taken up by the 10 mg. sample of gallotannin. On the basis of 5% of gallotannin impurity in the starches, the absorption of iodine thus displayed by the authentic sample is much more than enough to account for the excessive iodine absorption by the wood starch samples during mol.wt. determinations by hypoiodite oxidation.

Since the presence of mere traces of iodine-reactive impurities would be sufficient to upset mol. wt. determinations by hypoiodite oxidation, the colorimetric 3:5 dinitrosalicylic acid method of Meyer (166) was resorted to. To reduce the possibility of inter-reaction between this reagent and the wood-starch impurities, it was decided to use the samples recovered

from 0·5N sodium hydroxide solution through which air had been bubbled for some 20 hours to oxidise the contained gallotannin. The alkaline suspensions of these samples prior to the addition of the 3:5 dinitrosalicylic acid solution possessed varying shades of brown coloration. These inherent colorations, however, were adequately compensated for in the subsequent colorimetric estimation by filling the 'control cell' of the Spekker absorptiometer with solutions containing the respective samples in concentrations identical with those of the actual experimental solutions. The mol.wt. values thus obtained are given in the following table.

Mol.Wts. of Wood Starches by Method of K.H. Meyer (166).

| Source. | Mol.Wt. | D.P. |
|---|---|---|
| Elm sapwood | $1·2 \times 10^5$ | 740 |
| Maple " | $1·02 \times 10^5$ | 630 |
| Oak " | $1·43 \times 10^5$ | 880 |
| Turkey Oak sapwood | $1·34 \times 10^5$ | 830. |

TABLE VII.

It will be observed that these values are considerably lower than the corresponding values for potato and waxy maize starches given in Table V. It may well be that the persistence of a reactive impurity is responsible for the relatively lower values given by the wood starches. In the case of elm starch,

at least, this seems very likely, since viscosimetric data for the methylated product in m-cresol solution indicate a mol.wt. of the order of $5 \times 10^5$.

Acetylation of Wood Starches.

Oak starch dispersed in pyridine was acetylated in the presence of acetic anhydride, the temperature being maintained at about 20° for two days. The acetylated product was isolated by precipitation into water and was recovered as a friable, brown-coloured powder which gave no colour with iodine. It was found to be insoluble in ether, alcohol, chloroform, acetone and m-cresol, although in the latter three solvents it swelled greatly to a semi-transparent, gelatinous mass which did not undergo further dissolution in these solvents on standing for two months. Hirst, Plant and Wilkinson (23) reported that potato starch acetate prepared in pyridine medium was similarly insoluble. Kerr and Cleveland (231) recently suggested that the limiting D.P. for solubility in chloroform, at least for amylose acetates, is of the order of 1100 glucose residues.

"Prepared" elm and oak starches were each readily acetylated with a mixture of acetic anhydride and glacial acetic acid in the presence of thionyl chloride as catalyst (22). The impurities, in each case, remained suspended as greenish-brown precipitates in

the solution from which they were separated at the centrifuge. The acetylated starches were isolated from the clear centrifugates as pure-white powders which were relatively easily soluble in chloroform and acetone. Their specific rotations in chloroform solution were found to conform roughly with those of other acetylated starches and their mol.wts. as determined viscometrically, appeared to be of the order of $2 \times 10^5$.

In order to obtain some idea of the extent of degradation brought about by acetylation in acetic acid - acetic anhydride medium using thionyl chloride as catalyst, the sample of oak starch (D.P. 160), which had been degraded by treatment with 0·5% ethanolic-hydrogen chloride was acetylated in this manner. The material dissolved quickly and completely in the acetylating medium and was finally recovered as a white powder readily soluble in cold chloroform and cold acetone. Its apparent mol.wt. as determined viscometrically in m-cresol solution was approximately $6 \times 10^4$ (D.P. $\simeq$ 210). The product was deacetylated by treatment with sodium methoxide and the free starch recovered as a white powder, B.V. 0·171, corresponding with 9·3% of amylose. Potentiometric iodine titration of the sample indicated the presence of 9·5% of amylose as compared with the corresponding value of 14·2% amylose for the sample prior to acetylation. The

amylose in the latter sample adsorbed iodine during potentiometric titration at an appreciably lower potential than that of the former. It thus seems that acetylation with the above reagents had brought about some degradation of the amylose component and, therefore, in all probability, of the amylopectin component also (216). Degradation could not have been unduly extensive, however, since the apparent D.'s P. of the sample before acetylation and after acetylation followed by deacetylation were, respectively, 160 and 156 as determined by colorimetric estimation of the products of reduction of 3:5 dinitrosalicylic acid (166).

End-group Assay on Methylated Elm Starch.

Granular elm starch was methylated directly in the absence of air by the repeated application of dimethyl sulphate and concentrated sodium hydroxide solution. The methoxyl content of the product could not be increased beyond 44·1% by continued treatment with these reagents. Gallotannin and other coloured impurities had obviously been removed during methylation and the product possessed a specific rotation (viz. $[\alpha]_D$ + 208°) and a specific viscosity in m-cresol (viz. $\eta_{sp/c}^{20}$ = 3) which were comparable with those of other similarly methylated starches.

Hydrolysis of methylated elm starch and separation of the products on a paper chromatogram indicated the

presence of tetramethylglucopyranose, 2:3:6 trimethylglucose and two dimethylglucoses as well as faint traces of a monomethylglucose and of glucose itself. Quantitative estimation of each of the major products of hydrolysis separated on a paper chromatogram was accomplished by the micro-technique described by Hirst, Hough and Jones (232). The results showed that of the total weight of the mixed methylated sugars obtained by hydrolysis of the methylated amylopectin component, some 5·3% was represented by tetramethylglucose. This means that the repeating-unit of elm sapwood amylopectin consists of about 20 anhydroglucose units.

Larger scale separation of the components of the hydrolysate of methylated elm starch was effected chromatographically on a cellulose column using the techniques described by Hough, Jones and Wadman (233). Calculations based upon the yield of tetramethylglucose thus obtained and also upon the assumption that preferential loss of one or the other component of the 'whole' starch did not occur during methylation, confirmed that the amylopectin repeating-units consisted, on the average, of 20 $\pm$ 1 anhydroglucose units.

The sample of oak starch which had been bleached by treatment with chlorine and sulphur dioxide was also methylated and again it was found that the methoxyl

content could not be increased beyond 44·0%. That this sample had suffered dextrinisation, probably by the hydrolytic activity of the bleaching agents, was indicated by the relatively low specific viscosity in m-cresol of its methyl derivative. An apparent mol.wt. of about $6 \times 10^4$ (D.P. $\simeq$ 290) was calculated from the viscosity data. Quantitative hypoiodite estimation of the hydrolysis products of a small sample of the methylated starch separated on a paper chromatogram (232) indicated that the repeating-unit of the amylopectin component consisted of some 12 anhydroglucose units only. Further evidence that the original sample was degraded was the relatively high potential at which the amylose component adsorbed iodine during potentiometric iodine titration. The methylated product was, therefore, not further investigated.

General Conclusions. From the foregoing, it appears that wood starches are constituted similarly to the majority of the known varieties of starch. Many of the methods and techniques usually employed for the investigation of starch and polysaccharides in general provide misleading results when applied directly to the wood starches because of the reactivity of the associated impurities which have been found difficult to remove by methods which do not bring about simultaneous

degradation of the starches. In some cases, however, reliable compensation can be made for the effect of these impurities and the true behaviour of the starches can thus be determined beyond reasonable doubt. The evidence obtained from an examination of the products of hydrolysis of the methylated starches appears to be incontrovertible and this technique seems to provide the most reliable method of attack on the problem.

The presence of a typical amylose component in the starches has been established by its isolation in spherocrystalline form as the butanol-complex, and it has been demonstrated that this component occurs to the extent of 14-20% in the various samples. The amylopectin component of elm starch has been shown to possess a proportion of non-reducing end-groups which approaches the upper limit of the range over which those of the known amylopectins extend. It is possible that elm amylopectin possesses at least one unusual feature in that the proportion of inter-unit bonds of the 1:6 variety (viz. 35%) appears to be lower than the corresponding values for several other amylopectins previously investigated.

## CHAPTER III.

## E X P E R I M E N T A L.

Unless otherwise stated, the drying of solids prior to the various analyses described in the following pages was performed by heating at 60° *in vacuo* over phosphorus pentoxide for 8-12 hours.

*Tests Applied for the Identification of the Impurities Associated with the Wood Starch Samples.* Owing to the fact that the samples were available in limited quantities only, very little of each could be reserved for the following tests. These tests, therefore, are not really comprehensive, but they do serve to indicate the nature of the impurities present.

1). *Anthocyanin(s) in Elm and Oak Starches.* The granules (0·2 g.) were treated for 30 seconds with boiling ethyl alcoholic-hydrochloric acid (10 ml.;1%) and the mixture allowed to cool during 30 minutes. The pink colour which developed was replaced by a green colour on making the solution alkaline by the addition of sodium hydroxide solution (235,210).

2). *Lignin in the Residues Remaining After Hydrolysis of the Starches.* Maiile (223) prescribed the following test for lignin. Chlorine gas is bubbled slowly for 30 seconds through an aqueous suspension (ca. 5%) of the suspected lignin. Sulphurous acid is then added to destroy the excess of chlorine followed by sufficient sodium sulphite to render the solution

distinctly alkaline. The development of a pink colour represents a positive test for lignin.

It was found, however, that gallotannin and gallic acid also give strongly positive reactions to this test. For this reason, the insoluble brown residues left after sulphuric acid hydrolyses of the starch samples (see later) were freed as far as possible from gallotannin by continued boiling for 6 hours with sulphuric acid (5%) followed by prolonged washing with hot water. The insoluble brown impurities thus obtained from elm, oak and turkey oak starches were separately subjected to the above test to which they each gave positive reactions.

3). Gallotannin in the Aqueous Extract of Oak Sapwood. Oak sapwood sawdust was extracted as a suspension (2%) with boiling water for 2-3 minutes. The suspension was filtered to yield a clear, pale-brown, faintly acidic solution to which the following colour tests (236) for gallotannin were applied:-

(a) The addition of neutral ferric chloride solution gave an olive-green colour. (The development of a blue-black colour or precipitate or a greenish colour is said to confirm the presence of gallotannin).

(b) The addition of lime-water gave a faint white precipitate. (Gallotannin with this reagent gives a white precipitate which changes first to blue and then

to blue-green on addition of excess of the reagent).

(c) The addition of dilute ammoniacal potassium ferricyanide solution gave a red-brown colour which further confirmed the presence of gallotannin.

(d) The application to the infusion of the αα' dipyridyl test described by Feigl (237) resulted in the formation of a flocculent violet precipitate. This is claimed to be a sensitive and selective test for gallotannin.

These tests established the presence of gallotannin in the aqueous infusion of oak sapwood. Although the tests were not applied directly to the actual starch granules, it seems highly probable that the latter adsorbed gallotannin from the aqueous medium during their extraction from the oak sapwood sawdust. This may be inferred from the qualitatively identical behaviour after atmospheric oxidation in alkaline solution of (i) the starch granules, (ii) the aqueous infusion of oak sapwood sawdust and (iii) an authentic sample of gallotannin (prepared by E. Merck, Darmstadt) towards potassium iodide. This effect is discussed in the previous chapter. Further support is provided by the fact that (i) the starch granules dispersed in water, (ii) the aqueous infusion of oak sawdust and (iii) aqueous solutions of gallotannin all react with free iodine as demonstrated, in each case, by the transient nature of the blue colour of the

starch-iodine complex.

4). Fibrous Material Admixed with the Granules of the Starch Samples. Microscopic examination revealed the presence of much fibrous material in a "second grade" sample of oak starch but of mere traces of fibre in the elm starch sample.

Uronic anhydride estimations were performed on both samples using the apparatus and method described by Swenson, McCready and McClay (227) except that 12% hydrochloric acid was substituted for 19% hydrochloric acid in the reaction flask and heating at 135-140° was continued for 4·5 hours. These modifications were made in order to perform the estimations under conditions as closely similar as possible to those employed by Campbell (224) for oak starch. The following data was obtained:-

Average Titre for Blank Experiments corresponded to 24·56 ml. 0·1N hydrochloric acid.

Titre obtained after decomposing dried elm starch (0·222 g.) corresponded to 24,10 mls. 0·1N hydrochloric acid. The liberation of $CO_2$ was, therefore, equivalent to 0·45% by weight of the starch sample used. This corresponds to an apparent uronic anhydride content of 1·80%.

Titre obtained after decomposing dried "second grade" oak starch (0·230 g.) corresponded to 23·56 ml. 0·1N hydrochloric acid. The liberation of $CO_2$ was

therefore, equivalent to 0·96% by weight of the starch sample used. This corresponds to an apparent uronic anhydride content of 3·84%.

Titre obtained after decomposing gallotannin (0·210 g.) corresponded to 23·60 ml. 0·1N hydrochloric acid. The liberation of $CO_2$ was therefore, equivalent to 1% by weight of the gallotannin used.

Moisture Contents of the Wood Starch Samples.

Portions of each sample were weighed before and after thorough drying to constant weight under the conditions described at the beginning of this chapter. The difference between the weights, in each case, was calculated as a percentage moisture content for each sample. The values thus obtained are as follows: elm starch 12·20, maple starch 14·76, turkey oak starch 14·00, oak starch 13·42, and degraded oak starch 14·10%.

Analyses of the Samples for Actual Starch Content.

The samples were hydrolysed as described below and the liberated glucose estimated by:-

1). The Use of Somogyi's Copper Reagent (222).

In each case the undried starch samples (10-12 mg.) were hydrolysed by heating at 100° with sulphuric acid (0.5 ml.; 5%) in a sealed tube for 6-7 hours. After thorough cooling, each tube was opened and the contents neutralised by the addition of solid barium carbonate. The neutralised hydrolysates were then filtered into

graduated flasks (250 ml.), the residues being washed well with water, and the volumes, in each case, finally made up to 250 ml. The weights of reducing sugar, calculated as glucose, were then estimated in portions of 5 ml. of each of these solutions using the micro-copper reagent described by Somogyi (loc.cit.) and using as a standard 5 ml. of a glucose solution the concentration of which was roughly similar to that of the "unknown" solutions.

The theoretical conversion factor (viz. 0·9) was employed for the calculation of the weight of actual starch in the original samples from the weight of glucose in the hydrolysates estimated by the above technique.

The following actual starch contents were thus estimated for the various samples: elm starch 78·5, maple starch 75·5, turkey oak starch 76·1, oak starch 78·8, and degraded oak starch 75·0%.

2). Alkaline Hypoiodite oxidation.

In the cases of turkey oak, oak and degraded oak starches, the undried samples (0·12 - 0·14 g.) were each hydrolysed by heating at 100° with sulphuric acid (10 ml.; 5%) under reflux for 6-7 hours. Sodium hydroxide solution (4%) was then added until the solutions were neutral to phenol red (0·05 ml. of 0·04% being added). The neutralised hydrolysates were then freed by filtration from the insoluble brown residues

of lignin (?) and the volumes of the filtrates in each case made up to approximately 200 ml. with water. Sodium hydroxide (35 ml.; 0·1N) was then added to each followed by iodine solution (20 ml.; 0·1N). The mixtures were allowed to stand undisturbed in the dark for 75 minutes and then acidified by the addition of sulphuric acid (3 ml.; 2N). The excess iodine in each case was then titrated with standard sodium thiosulphate solution (0·1N). The results of these titrations, together with the result of a 'blank' experiment, permitted the calculation of the weight of reducing sugar (calculated as glucose) which was present in each of the hydrolysates. The starch contents of the samples were then calculated as before, the values being given below.

The brown residues which resulted from the hydrolyses of the samples and which were separated by filtration prior to hypoiodite oxidation of the hydrolysates were transferred separately to a Gooch crucible and each dried to constant weight. These weights, expressed as percentages of the weights of the respective undried starch samples are incorporated in the following data as "lignin" contents: turkey oak starch 74·0%, lignin 8·1%; oak starch 73·2%, lignin 5·8%; degraded oak starch 73·0%, lignin 5·6%.

3). <u>Separation on a Paper Chromatogram and Quantitative Estimation of the Glucose formed by Hydrolysis of Elm</u>

Starch. The undried elm starch sample (11·31 mg.) was hydrolysed by heating at 100° with sulphuric acid (0·5 ml.; 5%) in a sealed tube for 6-7 hours. The tube was then opened and D-ribose (11·39 mg.) was added to the acid hydrolysate which was then neutralised by the addition of solid barium carbonate. The mixture was centrifuged and a portion of the clear supernatant solution spotted on to the starting-line of a quantitative paper chromatogram which was then irrigated with the upper phase resulting from the separation of a well-shaken mixture of n-butanol (40 ml.), ethanol (10 ml.) and water (50 ml.) containing 2% concentrated ammonia solution under the conditions prescribed by Partridge (218). The glucose and ribose thus separated were estimated according to the directions of Flood, Hirst and Jones (186), the following data being obtained.

| | | | | | | |
|---|---|---|---|---|---|---|
| Titre for | "water blank" | 5·653 | ml. | 0·005N | sod.thiosulphate. | |
| " | "ribose paper blank" | 5.398 | " | " | " | " |
| " | "glucose paper blank" | 5.447 | " | " | " | " |
| " | "standard"ribose | 4.469 | " | " | " | " |
| " | " glucose | 4.041 | " | " | " | " |
| " | "unknown"ribose | 4·118 | " | " | " | " |
| " | " glucose | 3·903 | " | " | " | " |

The "standard" ribose solution contained 43·54 mg.

D-ribose per litre.

The "standard" glucose solution contained 41·54 mg. D-glucose per litre.

Calculations based on this data indicate that the sample of elm starch contained 76·6% of actual starch.

Determination of Specific Rotation of Elm Starch Hydrolysate. Undried elm starch (0·1154 g.) was hydrolysed by heating at 100° with sulphuric acid (10 ml.; 5%) under reflux for 7 hours. The insoluble red-brown residue was collected in a weighed Gooch crucible and dried to constant weight (0·0053 g., i.e. 4·6% of the weight of the undried starch sample).

The optical rotation of the filtrate was calculated as the specific rotation of the theoretical yield of glucose and found to be $[\alpha]_D^{19}$ + 51°.

General Properties of the Wood Starch Samples.

Weak pastes of the undegraded wood starches with water gave deep blue colorations with iodine solution (0·2% in potassium iodide solution).

The samples were prepared for blue-value determinations by dispersing appropriate weights of each (containing exactly 10 mg. actual starch) in water (1 ml.) to which sodium hydroxide solution (0·2 ml.; 10%) was added. The samples were allowed to disperse at room temperature overnight and the volumes then made up to 10 ml. in each case with water. The dispersions were centrifuged and 5 ml. portions of each

were further prepared according to the directions of Hassid and McCready (94). The blue-values were then determined at 18° as described by Bourne and Peat (100) with the following results: elm starch 0·337, maple starch 0·176, turkey oak starch 0·283, oak starch 0·306, and degraded oak starch 0·161. Under similar conditions potato starch gave a blue-value of 0·288.

Aqueous dispersions of the wood starches had no obvious effect on Fehling's solution in the cold, but on boiling, the dispersions became greenish-brown in colour and slight greenish-brown precipitates formed. This effect was possibly due to the gallotannin impurity in the starches, since a 5% aqueous solution of the authentic sample of this substance was found to give a green coloration in the cold with Fehling's solution. Gradual heating of the mixture to the boiling-point produced a heavy, flocculent green-brown precipitate which soon distintegrated to yield a fine, deep red-brown precipitate.

The wood starches dispersed in sodium hydroxide solution with somewhat more difficulty than either potato or barley starches under identical conditions. Furthermore, the presence of the gallotannin impurity in the alkaline medium caused the wood starch dispersions to become deep brown in colour. For these reasons the optical rotations of the wood starches were determined in solution in perchloric acid (4·8N),

the optical rotation of elm starch only being determined also as a dispersion in sodium hydroxide solution. In each case, suspended impurities were first removed by centrifuging. The specific rotations of the various samples thus determined are as follows: elm starch $[\alpha]_D^{19°}$ + 149° (c, 0·21 in 0·5N sodium hydroxide), $[\alpha]_D^{17°}$ + 191° (c, 0·51 in 4·8N perchloric acid), maple starch $[\alpha]_D^{17°}$ + 185° (c, 0·49 in 4·8N perchloric acid), turkey oak starch $[\alpha]_D^{17°}$ + 188° (c, 0·48 in 4·8N perchloric acid), oak starch $[\alpha]_D^{17°}$ + 190° (c, 0·49 in 4·8N perchloric acid), degraded oak starch $[\alpha]_D^{17°}$ + 199° (c, 0·49 in 4·8N perchloric acid). For comparison, the specific rotation of potato starch in perchloric acid solution was also determined and found to be $[\alpha]_D^{17°}$ + 197° (c, 0·49 in 4·8N perchloric acid).

Potentiometric Iodine Titrations for the Determination of the Amylose Contents of Wood Starches. The method used was the modification elaborated by Wilson, Schoch and Hudson (128) of the method of Bates, French and Rundle (103). The experimental technique was briefly outlined in the previous chapter and the method of calculation of the final results from the data thus obtained has been reviewed in detail by Halsall (187). In each case, the amylose contents of the samples were calculated on the basis of the iodine uptake of 21·5% determined by Higginbotham and Morrison (104) for pure

amylose.

1). Apparent Amylose Content of Elm Starch.

A general description of the following experiments along with a discussion of their significance is given in the previous chapter.

(a) By potentiometric iodine titration of elm starch started 15 minutes after the addition of the potassium iodide (10 ml.; 0·5N) it was found that 0·022 g. of iodine was taken up by 1 g. of the starch. The apparent amylose content of elm starch was, therefore, 10·2% (see Fig.XXII).

(b) By potentiometric iodine titration of elm starch started 25 minutes after the addition of the potassium iodide (10 ml; 0·5N) it was found that 0·0155 g. of iodine was taken up by 1 g. of starch. The apparent amylose content of elm starch, in this case, was, therefore, 7·2% (see Fig.XXII).

Prior to subsequent potentiometric amylose determinations on wood starches, the potassium iodide (10 ml.; 0·5N) was added to the neutralised dispersions and the mixtures allowed to stand undisturbed for 1 hour. During this time the dispersions became purple in colour due to the liberation of free iodine. The volumes of sodium thiosulphate solution (0·001N) which were necessary to remove exactly this free iodine are given along with other relevant data for each of the starches.

(c) Sodium thiosulphate (5·44 ml.; 0·001N) was added to a dispersion of elm starch (19·94 mg.), and the volume made up to 100 ml. with water immediately prior to the potentiometric iodine titration. The amount of iodine taken up by 1 g. of starch was 0·044 g. The amylose content of elm starch was, therefore, 20·5% (see Fig.XXIII).

Dispersions of maple, turkey oak, oak and barley starches were prepared for potentiometric iodine titration in a manner similar to that described for elm starch under (c) above. The data for experiments on the former are given in tabular form below.

Amylose Contents of Wood Starches.

| Source of Starch. | Wt. of Sample | Volume of $Na_2S_2O_3$ (0·001N) added. | Wt. Iodine taken up by 1 g. Starch. | Amylose Content. |
|---|---|---|---|---|
| Maple | 32·78 mg. | 11·81 ml. | 0·0407 g. | 18·9%. |
| Turkey Oak | 32·08 mg. | 4·12 ml. | 0·0375 g. | 17·4%. |
| Oak | 40·04 mg. | 4·42 ml. | 0·0299 g. | 13·9%, |
| Oak (degraded) | 39·93 mg. | 4·05 ml. | 0·0265 g. | 12·3%. |
| Barley | 33·20 mg. | 7·25 ml. | 0·0400 g. | 18·6%. |

(NOTE: To the sample of barley starch (33·2 mg.), gallotannin (2·1 mg.) & finely divided lignin (2·5 mg.) had been added.)

TABLE VIII.

Fractionation of Oak and Elm Sapwood Starches.

(a) Granular Oak Starch (1·7 g. actual starch) was made into a paste with hot water (30 ml.) and added, with vigorous mechanical stirring, to a mixture of

pyridine (30 ml.) and water (40 ml.) at 92°. Stirring was continued and the temperature was maintained at 92° for 5·5 hours during which time almost complete dispersion of the oak starch occurred (102).

Water (100 ml.) at 92° was then added to dilute the pyridine content of the medium to 15% (102). The dispersion was centrifuged while hot to remove undissolved impurities and undispersed granule fragments which were then washed with alcohol and ether, and dried to yield a horny, brown residue (0·2 g.).

The clear, brown, slightly viscous centrifugate was allowed to cool from 90° in a Dewar vessel. The liquid had cooled to room temperature within two days but little precipitation occurred during this period and the mixture was allowed to stand undisturbed for a further 2 days before separating the sparse precipitate at the centrifuge. The almost white residue (Fraction I) consisting of the amylose-pyridine complex was washed well with warm alcohol and dried <u>in vacuo</u> first over calcium chloride and then over phosphorus pentoxide for 2 days. Yield of crude oak amylose, 0·11 g., B.V., 0·954.

The supernatant liquid from the above centrifugation was allowed to stand undisturbed at room temperature for a further 2 days when a further quite bulky precipitate appeared (Fraction II). This was removed at the centrifuge, the pyridine complex being decomposed

and the crude amylose dried as for Fraction I. Yield of Fraction II, 0·21 g., B.V. 0·895.

The centrifugate after the removal of Fraction II was evaporated under reduced pressure at 40° to a volume of about 25 ml. The concentrate was filtered into alcohol (300 ml.) with manual stirring and the precipitated crude amylopectin fraction isolated at the centrifuge. It was washed several times with alcohol and dried *in vacuo* first over calcium chloride and then over phosphorus pentoxide at 60° for 3 hours. Yield, 1·24 g., B.V. 0·14.

Crude amylose Fractions I and II were combined and were found to be only partially soluble in cold water. The solution gave an intense pure blue colour with a little iodine. In hot water, retrogradation typical of that shown by the pure amylose components of other starches occurred. That is, a tough, transparent film developed across the surface of the solution (87). This film was easily removed intact and washed. It was found to be completely insoluble in hot and cold water.

Before proceeding to the purification of the crude oak amylose it was necessary to convert it to an easily soluble form. This was effected by the so-called "rejuvenation process" described by Bernfeld and Studer-Pécha (238) by mixing the retrograded amylose with water (2-3 ml.) and adding sodium

hydroxide solution (5 ml.; 2N). The mixture was stirred vigorously and the temperature maintained at 50° for 2 hours. The solution was centrifuged and the opalescent supernatant liquid was neutralised by the addition of acetic acid and then poured with stirring into alcohol (200 ml.). The flocculent precipitate was isolated by centrifuging and the residue of crude amylose was dispersed immediately in butanol-saturated water (200 ml.) at 90° (102). The dispersion was centrifuged while hot, and cooled from 90° to room temperature over 15 hours in a flask well-lagged with cotton-wool. After further cooling for an hour or two at 3-4°, the amylose-butanol complex was separated at the centrifuge and the white residue washed three times with butanol-saturated water, centrifuging between washings. The clear centrifugates obtained each time gave greenish-blue or red-blue colours with iodine, presumably due to the extraction of small quantities of the amylopectin component.

The residue from the final washing and centrifuging was redispersed for 20 minutes in butanol-saturated water (200 ml.) at 90°. The amylose-butanol complex precipitated on slow cooling of this dispersion was isolated and washed as before. The whole process was repeated a third time and it was observed that the second and third washings with butanol-saturated water

then apparently extracted little or none of the amylopectin component since the wash liquors, on these occasions, remained uncoloured on the addition of a little iodine.

The amylose-butanol complex from the last fractionation was examined under the microscope and found to consist of micro-spherocrystals similar to those described and photographed by Schoch (120). The crystals of oak amylose-butanol complex appeared as small lobes regularly arranged in clusters of 2,3,4,5 and 6 lobes in 'daisy-petal' fashion.

The amylose-butanol complex was decomposed by adding water (50 ml.) and evaporating the mixture under reduced pressure at 35°. As the butanol was removed, the amylose dissolved in the water to form a clear solution which was ultimately evaporated to dryness. The residue of oak amylose (0·14 g.) represented only 60% of the theoretical yield based on the amylose content of 13·9% for the original starch. Blue-value of the sample, 1.20; $[\alpha]_D^{19^\circ}$ + 192° (c, 0·48 in water) c.f. $[\alpha]_D$ + 195-7° reported by Meyer, Bernfeld and Wolff (127) for maize amylose.

Potentiometric Iodine Titration of Oak Amylose.

A dispersion of thesample (5.88 mg.) was prepared for the potentiometric titration as for 'whole' starches (103,128). On the addition of potassium iodide (10 ml.; 0·5N) the persistence of gallotannin in the amylose

preparation became obvious by the development and gradual deepening of a blue colour in the dispersion. After one hour, sodium thiosulphate (3 ml.; 0·0005N) was added until the blue colour was just destroyed. The volume was made up to 100 ml. with water and the potentiometric iodine titration carried out in the normal manner. The amount of iodine taken up by 1 g. of the amylose sample was 0·191 g. If 21·5% is the amount of iodine taken up by pure amylose (104), the amylose content of the oak amylose sample was 88·8%.

(b) Granular Elm Starch (1·7 g.) was dispersed in 30% pyridine according to the directions for oak starch above, except that heating at 92° was continued for 8 hours. Some difficulty was experienced in dispersing the sample of elm starch and even after treatment for 8 hours with the hot 30% pyridine, a bulky, dark-brown residue (0·38 g.) remained after centrifuging the hot dispersion. This residue partially dissolved in dilute perchloric acid solution and the solution gave a blue colour with iodine.

The clear dispersion in 15% pyridine at 92° was allowed to cool undisturbed in a Dewar vessel for 6·5 days. The precipitated amylose-pyridine complex which was brown in colour was isolated as previously described. The moist elm amylose complex was dispersed in butanol-saturated water at 92°,

reprecipitated and washed with butanol-saturated water. Three reprecipitations involving nine washings yielded a product which was pale-brown in colour and which, on microscopic examination, was found to exist in spherocrystalline form similar in all respects to the oak amylose-butanol complex described above. Water (50 ml.) was added to the complex and the mixture evaporated under reduced pressure at 35° until the last traces of butanol were removed. The slightly opalescent solution was made up to a volume of exactly 50 ml. with water and an aliquot portion (5 ml.) of this solution was removed and the amylose hydrolysed by adding concentrated sulphuric acid (0·2 ml.) and heating under reflux at 100° for 10 hours. The hydrolysate was cooled and neutralised with solid barium carbonate and filtered into a graduated flask (500 ml.). The residue was washed well with water and the volume made up to 500 ml. The weight of glucose in portions (5 ml.) of this solution was then estimated by means of Somogyi's copper reagent (222). Calculations based on the result indicated that the final yield of elm amylose was 0·1912 g. representing 55% of the theoretical yield; $[\alpha]_D^{17^\circ}$ + 194° (c, 0·52 in water), B.V. 1·22.

Potentiometric Iodine Titration of Elm Amylose.

A portion of the aqueous solution (3 ml.)

containing elm amylose (11·47 mg.) was treated with potassium hydroxide (10 ml.; 0·5N) for 4-5 hours in an attempt to oxidise completely the associated gallotannin. The solution was then neutralised and potassium iodide (10 ml.; 0·5N) added. The solution quickly became purple in colour. After 1 hour the colour was just discharged by the addition of sodium thiosulphate (6 ml.; 0·0005N). The volume was made up to 100 ml. with water and the potentiometric iodine titration then conducted in the normal manner. The amount of iodine taken up by 1 g. of the amylose sample was 0·196 g. The amylose content of the sample was therefore, 91·2%.

Periodate Oxidation of Granular Elm Sapwood Starch.

Granular elm starch (0·2365 g. of actual starch) was suspended in water (52·5 ml.) containing potassium chloride (2·5 g.) and sodium metaperiodate solution (7·5 ml.; 0·293M) was added (178,187). The mixture was gently shaken in a glass-stoppered bottle in the absence of light for 14 days at room temperature. At intervals after oxidation for 160 hours under these conditions, shaking was temporarily discontinued and the suspended solids allowed to settle, on each occasion, during 4-5 hours. Samples (10 ml.) of the clear supernatant solution were withdrawn each time and ethylene glycol (0·5 ml.) was added to each to destroy the periodate. The samples were then titrated

against sodium hydroxide solution (0·01N) using methyl red as indicator.

After the removal of each sample the shaking was continued.

A blank experiment was performed simultaneously by shaking a mixture prepared by adding sodium metaperiodate solution (7·5 ml.; 0·293M) to water (52·5 ml.) containing potassium chloride (2·5 g.). Shaking of this mixture was interrupted at the same time intervals as the actual experimental mixture and samples (10 ml.) of this 'blank' solution were also withdrawn and titrated against sodium hydroxide solution (0·01N) after the addition of ethylene glycol (0·5 ml.). The results of the blank experiment thus enabled correction of the experimental titres for the effect of the periodate reagents.

In the cases of the second, third and fourth experimental titres a further correction was applied to allow for the fact that the oxidised starch granules, being insoluble, were not removed from the bottle when the samples of supernatant liquid were withdrawn for titration.

<u>"Corrected" Titres</u>. 2·804 ml. 0·01N sodium hydroxide solution (167 hours); 2·881 ml. (185 hours); 3·014 ml. (258 hours); 3·023 ml. (310 hours). These titres were plotted against time and the acid yield after periodate oxidation for 150 hours as indicated by the

graph, corresponded to the formation of 7·7 mg. formic acid from the starch, i.e. to the presence of one non-reducing terminal residue per 7 glucose units in the amylopectin component on the basis that the 'whole' starch contained 20·5% of amylose.

Determination of Glucose Residues in Elm Amylopectin Linked through $C_1$, $C_4$, and $C_6$ (27,185,187).

Shaking of the suspension (20 ml.) containing the oxidised elm starch from the previous experiment was continued for a total of 14 days. Excess of ethylene glycol (1·5 ml.) was then added and shaking continued for a further 24 hours. The oxidised starch was filtered on a G4 sintered-glass filter and washed with cold water until free from iodate (i.e. until the filtrate gave negative tests with potassium iodide and sulphuric acid and with diphenylamine and sulphuric acid). The granules which still retained their buff coloration were then washed with alcohol (100 ml.) and dried in a vacuum desiccator over calcium chloride for 5 days. The dry, oxidised starch was then hydrolysed by heating at 100° with sulphuric acid (30 ml.; 0·5N) for 10 hours. A copious, flocculent, brown residue remained suspended in the brown-coloured hydrolysate. D-Ribose (12·62 mg.) was dissolved in the cold acid hydrolysate which was then extracted five times with portions (30-40 ml.) of ether. The solution was transferred quantitatively to a centrifuge tube where

it was neutralised in the presence of solid barium carbonate with occasional stirring until Congo red paper was no longer turned blue by contact with the supernatant liquid. The mixture was then centrifuged and the clear supernatant liquid siphoned into a flask from which it was evaporated to dryness under reduced pressure at 40°. The residue was dissolved in water (0·5 ml.) and portions of the solution transferred to the starting-lines of two quantitative paper chromatograms which were then irrigated with the upper phase resulting from the separation of a well-shaken mixture of n-butanol (40 ml.), ethanol (10 ml.) and water (50 ml.) containing 2% concentrated ammonia solution under the conditions prescribed by Partridge (218). The glucose and ribose thus separated were estimated by the techniques described by Flood, Hirst and Jones (186), the following data being obtained. The titres are given as millilitres of sodium thiosulphate (0·005N).

| Titres for:- | Estimation 1. | Estimation 2. |
|---|---|---|
| Water blank | 5·399 ml. | 4·835 ml. |
| Ribose paper blank | 5·230 " | 4·752 " |
| Glucose " " | 5·263 " | 4·756 " |
| "Standard" ribose | 3·120 " | 3·807 " |
| " glucose | 3·618 " | 4·043 " |
| "Unknown" ribose | 4·149 " | 3·250 " |
| " glucose | 4·448 " | 3·687 " |

Conc$^{n}$. of "Standard" Solutions:-

| | | |
|---|---|---|
| Ribose | 90·2 mg./l L. | 41·6 mg./l L. |
| Glucose | 49·7 mg./l L. | 24·1 mg./l L. |

The weights of glucose thus indicated in the hydrolysate of the sample of oxidised elm starch were 6·71 mg. and 6·75 mg. calculated from the data of estimations 1 and 2 respectively. The average of these results, namely 6·73 mg. glucose, representing 2·8% by weight of the actual elm starch oxidised, was used as a basis for the calculations made in the previous chapter.

Hydrolysis of Periodate-oxidised Gallotannin.

Gallotannin (90 mg.) was dissolved in water (60 ml.) in which solid potassium metaperiodate was suspended. A flocculent, red-brown precipitate formed immediately but dissolved on shaking the mixture for 4-5 hours to give a pale-yellow solution. The mixture was further gently shaken for 10 days after which it was filtered to remove the excess of solid potassium metaperiodate. The filtrate was evaporated under reduced pressure at 40° to remove a little volatile acidic material until the volume was reduced to 20 ml. The solution was suspended in a freezing mixture (-10°) for a short time during which much inorganic material crystallised out. This was removed by filtration and to the filtrate was added dilute ferric sulphate solution drop by drop until no further precipitation occurred. The solution was filtered and the filtrate evaporated to a volume of about 10 ml. when more inorganic material separated

and was removed. The solution was then evaporated to dryness to yield an orange-coloured, crisp solid (52 mg.) which was dissolved in water (2 ml.) to which ethylene glycol (0·1 ml.) was added. Sulphuric acid (15 ml.; 5%) was then added and the mixture heated at 100° under reflux for 10 hours during which time the solution became very dark in colour and iodine sublimed in the condenser. The hydrolysate was neutralised by the addition of solid barium carbonate and then filtered. The filtrate was shaken alternately with Amberlite Resins IR-100 and IR-4B to remove residual inorganic ions. The solution was evaporated to dryness and the residue dissolved in a little water (0·2 ml.). A small quantity of D-ribose was added to this solution and the contained sugars separated on paper chromatograms in the usual manner (218). The chromatograms were developed with ammoniacal silver nitrate and with aniline oxalate respectively and, in each case, small but definite spots were observed corresponding with an $R_G$ value of 0·14 calculated on the basis of the $R_G$ value for ribose of 0·21 reported by Hirst, Hough and Jones (232). An $R_G$ value of 0·14 was also estimated for glucose in a 'control' mixture of glucose and ribose separated on a paper chromatogram under conditions identical with those used in the above experiment.

Periodate Oxidation of Granular Maple Sapwood Starch.

Granular maple starch (0·2621 g. of actual starch) was suspended in water (52·5 ml.) containing potassium chloride (2·5 g.) and sodium metaperiodate solution (7·5 ml.; 0·293M) was added. The oxidation was carried out as in the case of granular elm starch. "Corrected" Titres: 3·362 ml. 0·01N sodium hydroxide solution (165 hours); 3·420 ml. (188 hours); 3·505 ml. (254 hours); 3·552 ml. (320 hours). These titres indicated the formation of 9·2 mg. of acid (calculated as formic acid) after oxidation for 150 hours, which corresponds to the formation of one mole of formic acid per 6·5 glucose residues in the amylopectin component of maple starch, on the basis that the 'whole' starch contained 18·9% of amylose.

Periodate Oxidation of Lignin. Various samples of lignin, namely, beech lignin, commercial 'alkali' lignin, Braun's 'native' spruce lignin and lignin isolated after hydrolysis of elm starch were separately treated with periodate under the conditions used for periodate oxidation of the wood starch samples. Only the results obtained on periodate oxidation of 'elm lignin' are recorded here since these are typical of the results obtained with the other lignin samples.

The lignin isolated after hydrolysis of elm starch was washed well with hot water and since the material

became horny on drying, a little of the wet sample (0·0215 g. calculated on the dry weight of the sample) was suspended in water (52·5 ml.) containing potassium chloride (2·5 g.), and sodium metaperiodate solution (7·5 ml.; 0·293M) was added. The experiment was continued as for the periodate oxidation of granular elm starch.

"Corrected" Titres: 0·289 ml. 0·01N sodium hydroxide solution (167 hours); 0·29 ml. (185 hours); 0·33 ml. (262 hours); 0·49 ml. (474 hours). If the acidic material generated by periodate oxidation of the lignin sample under these conditions be calculated in terms of formic acid, these titres indicate the formation of 0·77 mg. of formic acid after oxidation for 150 hours. The lignin content of the granular elm starch sample was shown to be 4·6%. On this basis, the lignin content (0·0142 g.) of the elm starch sample (0·3087 g.) periodate titration of which is described above, would have generated acidic material corresponding to 0·51 mg. of formic acid.

Periodate Oxidation of Gallotannin.

Gallotannin (0·0268 g.) dissolved in water (30 ml.) was neutralised to methyl red indicator by adding sodium hydroxide solution (1·9 ml.; 0·01N) and the volume of the solution made up to 52·5 ml. with water. Potassium chloride (2·5 g.) was added followed by sodium metaperiodate solution (7·5 ml.; 0·293M).

The oxidation was carried out as in the case of granular elm starch. The precipitated gallotannin-periodate complex ultimately dissolved, hence the titres were not "corrected."

Titres: 3·52 ml. 0·01N sodium hydroxide solution (167 hours); 3·56 ml. (186 hours); 3·60 ml. (257 hours); 3·76 ml. (330 hours). If the acidic material generated under these conditions be calculated in terms of formic acid, these titres indicate the formation of 9·7 mg. of formic acid after oxidation for 150 hours. Assuming the gallotannin content of elm starch to be 5%, the quantity of gallotannin (0·0154 g.) in the elm starch sample (0·3087 g.) oxidised with periodate may have liberated acidic material equivalent to 5·6 mg. formic acid.

Attempted Purification of Wood Starches:-

1). By Solution in Perchloric Acid (4·8N) (214). Granular elm starch (0·62 g.) was gelatinised by stirring with water (13·8 ml.) at 90° for 10 minutes. After cooling to 20°, the brown paste was treated with perchloric acid (16·2 ml.; 60%). Solution of the starch appeared to be complete within 20 minutes. After centrifuging to remove the insoluble dark-brown impurities (0.045 g.; 7·3% by weight of the starch sample), the solution was neutralised by adding sodium hydroxide solution (2N) and dialysed for 2 days. The dialysate was poured into alcohol (1000 ml.) and the

precipitated starch centrifuged, washed twice with alcohol and dried. Yield 0·43 g. (92% of theoretical), B.V. 0·34. Hydrolysis of the starch (10·52 mg.) was effected by heating in a sealed tube at 100° with a mixture of hydrochloric acid (0·5 ml.; 2N) and glacial acetic acid (0·2 ml.) for 7 hours. The liberated glucose was estimated as described previously with Somogyi's copper reagent (222). The result indicated that the recovered elm starch consisted of 89% of actual starch. Periodate oxidation: recovered elm starch (0·3025 g. of actual starch) was oxidised with periodate under conditions identical with those for granular elm starch. "Corrected" titres: 1·839 ml. 0·01N sodium hydroxide (168 hours); 1·887 ml. (195 hours); 1·94 ml. (242 hours); 2·072 ml. (340 hours). These titres indicated the formation of ca. 5 mg. of acid (as formic acid) after 150 hours oxidation, which corresponds to the formation of one mole of acid per 13-14 glucose residues in the amylopectin component.

Periodate Oxidations of:-

(a) Dried granular potato starch (0·3019 g.) containing 18% of amylose was oxidised with periodate under conditions identical with those for granular elm starch. "Corrected" titres: 1·083 ml. 0·01N sodium hydroxide solution (167 hours); 1·122 ml. (188 hours); 1·164 ml. (252 hours); 1·213 ml. (312 hours). These titres indicated the formation of 2·9 mg. of formic acid after

150 hours oxidation, which corresponds to the formation of one mole of acid per 24 glucose residues in the amylopectin component.

(b) Potato starch (0·2953 g. recovered from solution in 4·8N perchloric acid as described above for elm starch) was shaken as a suspension in water (20 ml.) for 24 hours. The suspension was neutralised to methyl red indicator by adding hydrochloric acid (2·14 ml.; 0·01N). Oxidation with periodate was then conducted under conditions identical with those used for granular elm starch. "Corrected" titres: 1·083 ml. 0·01N sodium hydroxide solution (172 hours); 1·120 ml. (197 hours); 1·179 ml. (269 hours); 1·230 ml. (342 hours). These titres indicated the formation of 2·95 mg. of formic acid after 150 hours oxidation which corresponds to the formation of one mole of acid per 23 glucose residues in the amylopectin component.

(c) Dried granular 'waxy' maize starch (0·2908 g.) which had been "defatted" by extraction with boiling 85% methanol (77) was oxidised with periodate under conditions identical with previous oxidations. "Corrected" titres: 1·37 ml. 0·01N sodium hydroxide solution (167 hours); 1·407 ml. (187 hours); 1·467 ml. (264 hours); 1·527 ml. (336 hours). These titres indicated the formation of 3·75 mg. of formic acid after 150 hours oxidation, which corresponds to the formation of one mole of acid per 22 glucose residues.

(d) 'Waxy' maize starch (0·3064 g. recovered from solution in 4·8N perchloric acid as described above for elm starch) was shaken with water (20 ml.) for 10 hours. The dispersion was neutralised to methyl red indicator by adding hydrochloric acid (3·65 ml.; 0·01N). Oxidation with periodate was then conducted as for previous oxidations. Since the recovered amylopectin was soluble the titres required no correction. Titres: 1·575 ml. 0·01N sodium hydroxide solution (173 hours); 1·642 ml. (196 hours); 1·824 ml. (270 hours); 1·981 ml. (343 hours). These titres indicated the formation of 4·25 mg. of formic acid after 150 hours oxidation, which corresponds to the formation of one mole of acid per 20-21 glucose residues.

2). By Treatment with Ethanolic Hydrogen Chloride.

Granular oak starch (3 g. of actual starch) was treated for 30 minutes with boiling absolute ethyl alcohol (10 ml.) containing hydrogen chloride (0·5% by weight of ethanol) according to the directions of Baird, Haworth and Hirst (215). The granules which retained their buff coloration were recovered by filtration and washed well with water. They dispersed readily in water (15 ml.) at 80° to form a mobile, slightly opalescent solution, leaving a suspension of dark-brown impurities which were removed by filtration. The clear filtrate was poured into alcohol (200 ml.)

and the precipitated, snow-white starch collected at the centrifuge, washed with alcohol and dried. Yield, theoretical; B.V. 0·23. Potentiometric iodine titration of the recovered starch (21·54 mg.) was conducted in the normal manner (103,128). The addition of potassium iodide (10 ml.; 0·5N) to the neutral dispersion was not followed by the liberation of free iodine; this suggested the absence of gallo-tannin in the sample. The amount of iodine taken up by 1 g. of starch was 0·0305 g. indicating that the amylose content of the sample was 14·2%.

Periodate oxidation: recovered oak starch (0·2938 g.) was oxidised with periodate under conditions identical with previous oxidations. Since the recovered starch was soluble the titres required no correction.

Titres: 2·159 ml. 0·01N sodium hydroxide solution (168 hours); 2·266 ml. (192 hours); 2·447 ml. (245 hours); 2·793 ml. (322 hours).

Periodate oxidation of potato starch (0·2979 g. recovered after treatment with 0·5% ethanolic-hydrogen chloride as described above for oak starch) under conditions identical with previous oxidations yielded the following data. Again, the titres required no correction. Titres: 2·325 ml. 0·01N sodium hydroxide solution (168 hours); 2·446 ml. (192 hours); 2·632 ml. (244 hours); 2·967 ml. (322 hours).

The interpretations of the latter two sets of

titres are given in the previous chapter.

3). By Decomposition of the Associated Gallotannin by atmospheric oxidation in alkaline medium according to the directions of Nierenstein and co-workers (211) except that sodium hydroxide solution (0·5N) was substituted for sodium bicarbonate solution (0·5N). Elm, maple, oak, turkey oak and potato starches (ca. 2 g. of each) were separately dispersed by frequent trituration over periods of 2-3 days in sodium hydroxide solution (50 ml.; 2N). The volume of each dispersion was made up to 200 ml. with water and air was bubbled through the dispersions as a continuous stream for about 20 hours, a little capryl alcohol (1 ml.) being added to prevent undue frothing. In each case, a fine, olive-green suspension was removed at the centrifuge and found to constitute some 7-9% of the starch samples. Glacial acetic acid was added to the clear amber-coloured supernatant liquids until the latter no longer turned red litmus paper blue. The neutralised dispersions were then dialysed for 2-3 weeks after which they were concentrated to volumes of 50-60 ml. by evaporation under reduced pressure at 40°, filtered through a G3 sintered-glass filter and poured with stirring into alcohol (500 ml.). The precipitated starches were collected at the centrifuge, washed with alcohol and dried in vacuo first over calcium chloride and then over phosphorus

pentoxide at 60°. Average Yields: 92-94% of theoretical.

Periodate Oxidation of the Recovered Starches.

Recovered potato starch (0·2985 g.) was shaken as a suspension in water (20 ml.) for 24 hours. The suspension was neutralised by adding hydrochloric acid (3·91 ml.; 0·01N). Oxidation with periodate was then conducted under conditions identical with those used for granular elm starch. "Corrected" titres: 1·053 ml. 0·01N sodium hydroxide solution (165 hours); 1·080 ml. (184 hours); 1·162 ml. (257 hours); 1·174 ml. (328 hours). These titres indicated the formation of 2·9 mg. of formic acid after 150 hours oxidation, which corresponds to the formation of one mole of acid per 24 glucose residues in the amylopectin component.

Periodate titration of the recovered wood starches was conducted similarly. The results are recorded in tabular form below.

| Source of Starch. | Wt. of Sample | Vol. HCl (0·01N) added to neutralise | "Corrected" Titres (0·01N. NaOH) | Liberated formic acid estimated at 150 h. | Calculated repeating-unit of amylopectin component. |
|---|---|---|---|---|---|
| Elm | 0·3192g. | 3·16 ml. | 1·501ml. (168h.)<br>1·570ml. (188h.)<br>1·651ml. (261h.)<br>1·703ml. (306h.) | 4·08 mg. | 17-18 |
| Maple | 0·3111g. | 3·92 ml. | 1·621ml. (168h.)<br>1·702ml. (189h.)<br>1·863ml. (261h.)<br>1·905ml. (306h.) | 4·44 mg. | 16 |
| Oak | 0·2991g. | 4·52 ml. | 1·486ml. (166h.)<br>1·578ml. (190h.)<br>1·669ml. (255h.)<br>1·695ml. (310h.) | 4·11 mg. | 18 |
| Turkey Oak | 0·3023g. | 4·21 ml. | 1·461ml. (167h.)<br>1·510ml. (189h.)<br>1·621ml. (261h.)<br>1·622ml. (307h.) | 4·06 mg. | 17-18 |

TABLE IX

Addendum: A Further Attempt to Purify Elm Sapwood Starch. Since the writing of the previous chapter, further efforts have been made to remove the gallo-tannin adsorbed on elm starch granules. As the previous attempts sometimes involved drastic treatment it was decided to re-investigate the possibility of solvent extraction of the impurity. The desired effect has now been achieved by extraction of the granules (0·8 g.) with boiling 85% aqueous methanol (50 ml.). Four successive extractions for 4-5 hours each with fresh portions of solvent removed 6·4% of solid material based on the weight of the granules extracted.

The extracted granules retained their buff coloration.

An aqueous solution of the material extracted from the granules possessed the following properties:-

1). The addition of a little neutral ferric chloride solution gave a greenish coloration.

2). A sparse reddish precipitate was obtained on application of the αα' dipyridyl test for gallotannin (237).

3). A brown colour developed on the addition of potassium hydroxide solution (0·5 ml.; 0·5N). After 30 minutes the solution was neutralised by the addition of dilute hydrochloric acid. A little starch paste was then added followed by potassium iodide solution. A purple colour soon developed and intensified on standing for 30 minutes when the solution was almost opaque. The colour was discharged on adding a few drops of dilute thiosulphate solution.

4). A little starch paste was added to a portion of the aqueous solution and iodine solution (0·05N) was added drop by drop. A transitory blue coloration developed and quickly faded until after the addition of the ninth drop of iodine solution which produced a blue colour which persisted for 5 minutes.

Periodate Oxidation; the extracted elm starch granules (0·3120g.) were oxidised with periodate under the usual conditions. In calculating the results it was assumed that the lignin impurity was not removed during

the above extraction and that this was responsible for the persistence of the buff coloration of the granules. It was calculated that the sample (0·3120 g.) used for periodate oxidation contained 0·2942 g. of actual starch, the remainder of the sample being represented by lignin.

"Corrected" titres: 1·482 ml. 0·01N sodium hydroxide solution (172 hours); 1·571 ml. (192 hours); 1·75 ml. (268 hours); 1·922 ml. (314 hours). These titres indicated the formation of 3·91 mg. of formic acid after oxidation for 150 hours which corresponds to the formation of 1 mole of acid per 17 glucose residues in the amylopectin component. However, on the basis of the results of periodate titration of the sample of lignin isolated after hydrolysis of elm starch, compensation can be made for the quantity of acidic material (equivalent to 0·64 mg. of formic acid) liberated by the lignin (0·0178 g.) contained in the above starch sample. This would mean that the amylopectin component of the sample was responsible for the liberation of 3·27 mg. of formic acid which corresponds to the formation of 1 mole of formic acid per 20 glucose residues in that component.

Acetylation of Wood Starches.

1). Acetylation of oak starch as a dispersion in pyridine (239,34). Oak starch (1 g. of actual starch) was gelatinised by stirring with water (50 ml.) at 90°

for 20 minutes. Redistilled pyridine (50 ml.) was added to the paste with vigorous stirring and the mixture evaporated under reduced pressure first at room temperature (14°) and later at 20-25° (bath temperature). As the azeotropic aqueous-pyridine mixture distilled off, more pyridine (280 ml.) was added drop by drop. The volume was finally reduced to 50-60 ml. when a thin brown jelly was obtained to which acetic anhydride (25 ml.) was added drop by drop with vigorous mechanical stirring, the temperature being maintained at 25°. Stirring was continued for 2·5 days at 18-20°. The mixture was then centrifuged from a little insoluble gelatinous material and the clear brown supernatant liquid poured with stirring into water (1000 ml.). The precipitated oak starch acetate was collected at the centrifuge, washed with water until free from acid, then with alcohol and ether, and dried in a vacuum over calcium chloride. Yield, 1·4 g. (79% of theoretical); Found: $CH_3CO$, 42·1%. (Calc. for $C_{12}H_{16}O_8$, 44·8%). The product was a brownish, friable solid insoluble in hot and cold water. Its aqueous suspensions gave no colour with iodine. It was also found to be insoluble in ether, alcohol, dioxan, acetone, chloroform and m-cresol, although it swelled greatly in the latter three solvents to give, in each case, a semi-transparent jelly.

2). Acetylation of oak starch with a mixture of acetic acid and acetic anhydride in the presence of thionyl chloride as catalyst (22). "Prepared" oak starch (1 g. of actual starch) was mixed with glacial acetic acid (6·5 ml.) through which chlorine gas had been bubbled for 30 seconds, i.e. until the liquid was distinctly yellow in colour. (In a previous experiment in which the acetic acid contained sufficient chlorine to impart only a pale-yellow colour, the starch did not dissolve subsequently in the acetylating medium after treatment for 6 hours). The mixture was stirred for 30 minutes at 20° and then acetic anhydride (22 ml. containing an amount of sulphur dioxide roughly equivalent to the chlorine in the glacial acetic acid) was added. Stirring at room temperature was continued for 1 hour after which the temperature was raised to 55°. After 2-3 hours at this temperature, the starch dissolved leaving a greenish-brown precipitate suspended in the medium in very finely divided form. This was removed at the centrifuge and the clear, colourless supernatant liquid poured with stirring into water (1000 ml.). The precipitated oak starch acetate was collected at the centrifuge, washed with water until free from acid, then with alcohol and ether, and dried in a vacuum over calcium chloride. Yield, 1·52 g. (86% of theoretical); $[\alpha]_D^{18^\circ}$ + 166° (c, 0·98 in chloroform); $\eta_{sp}^{20^\circ}$ 0·29 (c, 0·454 in m-cresol)

corresponding to an apparent mol.wt. of $1{\cdot}98 \times 10^5$ using Staudinger's equation with the value of the constant $k_m$ ($0{\cdot}93 \times 10^{-4}$) given by Staudinger and Husemann (37); (Found: $CH_3CO$, 45·2%. Calc. for $C_{12}H_{16}O_8$, 44·8%). The acetate was completely and easily soluble in acetone and chloroform and on evaporation of the solutions a clear, fragile film was formed.

3). <u>Acetylation of elm starch with a mixture of acetic acid and acetic anhydride in the presence of thionyl chloride as catalyst</u> (22). "Prepared" elm starch (0·62 g. of actual starch) was acetylated and the product isolated in a manner similar to that described above. A similar greenish-brown precipitate was removed after solution of the starch in the acetylating medium. Yield of white elm starch acetate, 0·91 g. (83% of theoretical); $[\alpha]_D^{17^\circ}$ + 167° (c, 1·0 in chloroform); $\eta_{sp}^{20^\circ}$ 0·32 (c, 0·47 in m-cresol) corresponding to an apparent mol.wt. of $2{\cdot}11 \times 10^5$; (Found: $CH_3CO$ 44·9%).

<u>Direct Methylation of Granular Elm Starch</u>. Granular elm starch (2·5 g. of actual starch) was made into a smooth cream with water (20 ml.) and sodium hydroxide (14 ml.; 30% by weight throughout) was added. The air in the flask was displaced by nitrogen, a steady stream of the gas being maintained throughout. Then sodium hydroxide (56 ml.; 30%) and dimethyl sulphate

(28 ml.) were added gradually at room temperature with vigorous mechanical stirring (35). After 15 hours' stirring at room temperature the mixture was again treated with sodium hydroxide (70 ml.; 30%) and dimethyl sulphate (28 ml.). A tendency for the mixture to froth was prevented by the addition of a little capryl alcohol. After 15 hours' stirring at room temperature, acetone (50 ml.) was added and the mixture was evaporated first on the steam-bath at atmospheric pressure and finally under reduced pressure (40 cm.) at 75°. As the acetone evaporated the partially methylated starch separated on the surface of the alkaline solution as an insoluble, spongy mass. The brown liquid was siphoned away from beneath the insoluble product and neutralised by the cautious addition of sulphuric acid (50%), with cooling in an ice-bath. The neutral solution was dialysed against running tap-water for 3 days and then evaporated under reduced pressure at 40° to a volume of 15-20 ml. This somewhat viscous solution was added to the bulk of the partially methylated starch dissolved in acetone (30 ml.) and the product remethylated by the gradual addition of sodium hydroxide (70 ml.; 30%) and dimethyl sulphate (28 ml.). After stirring for 15 hours, the product was isolated as described above, except that the hot alkaline liquor separated from the insoluble partially methylated starch was discarded on this and

subsequent occasions. This methylation procedure was repeated a further 11 times, the last 2 methylations being conducted at an elevated temperature (45°). The product finally isolated was washed 3 times with boiling water and then dissolved in acetone. The solution was separated from inorganic material at the centrifuge and then evaporated to dryness under reduced pressure at 30-35°. The residue was taken up in chloroform (150 ml.) and the solution filtered through a G3 sintered-glass filter. The filtrate was evaporated to a volume of 80 ml. and poured slowly with vigorous stirring into light petroleum (800 ml.; b.p. 60-80°). A pale-yellow oil separated which solidified on standing overnight. Yield, 2·86 g. (91% of theoretical) (Found: OMe 42·9%. Calc. for $C_9H_{16}O_5$, 45·6%).

Fractionation of the Methylated Starch. The methylated starch (2·85 g.) was dissolved in chloroform (100 ml.) and light petroleum (b.p. 40-60°) was slowly added with thorough mixing. After the addition of 600 ml. of petroleum, a pale-yellow oil separated (fraction I) which solidified after decantation of the supernatant liquid and the addition of fresh petroleum.

The bulk of the methylated starch in solution was precipitated by the addition of a further 100 ml. of light petroleum to yield fraction II, a pale-yellow oil which solidified under the same conditions as

fraction I.

Further slight precipitation occurred on the addition of more light petroleum to a total volume of 1000 ml. The oily precipitate was combined with the residue obtained on evaporation of the chloroform-light petroleum mixture to dryness. The product (fraction III) was discarded.

Found: Fraction I, 0·21 g.; $[\alpha]_D^{17^\circ}$ + 207° (c, 0·51 in chloroform); OMe, 43·7%; $\eta_{sp.}^{20^\circ}/c$ 3·26 (c, 0·41 in m-cresol), corresponding to an apparent mol.wt. of 494,000 (see Hirst and Young, ref.no.35). Fraction II, 2·48 g.; $[\alpha]_D^{17^\circ}$ + 208° (c, 0·88 in chloroform); OMe, 44·1%; $\eta_{sp.}^{20^\circ}/c$ 3·08 (c, 0·45 in m-cresol), corresponding to an apparent mol.wt. of 465,000. Fraction III, 0·08 g.

Fraction I was not further investigated.

Hydrolysis of methylated elm starch on a small scale and separation of the products on a paper chromatogram (232). Fraction II (52 mg.) was hydrolysed by heating at 100° with methanolic hydrogen chloride (1 ml.; 4%) in a sealed tube for 6 hours. After hydrolysis the tube and its contents were cooled and the tube opened cautiously. The methyl glycosides were then hydrolysed by heating at 100° with aqueous hydrochloric acid (5 ml.; 4%) for 6 hours. The hydrolysate was neutralised with silver carbonate and filtered. Silver ions were removed from the filtrate

by the passage of hydrogen sulphide. After further filtration, the solution was shaken alternately with Amberlite resins IR-100 and IR4B, and then evaporated to dryness under reduced pressure at 40° (Yield 43 mg. syrup). The syrup was dissolved in water (0·1 ml.) and the components of the syrup separated on a paper chromatogram (218) and quantitatively estimated by the method of Hirst, Hough and Jones (232). This method was modified slightly by conducting the alkaline hypoiodite oxidations of the methylated sugars in the presence of a sodium hydroxide-phosphate buffer (pH, 11·4) (240) in place of the carbonate-bicarbonate buffer (pH, 10·6). The qualitative paper chromatograms and the marginal strips of the quantitative chromatograms were developed with aniline oxalate.

| Sugar Indicated | Estimated $R_G$ value. | | Percentage by wt. of sugar in mixture. |
|---|---|---|---|
| 2:3:4:6-tetramethylglucose | 1·0 | | 4·3 |
| 2:3:6-trimethylglucose | 0·84 | | 86·5 |
| 2:3-dimethyl glucose | 0·60 | ) | |
| 2:6- " " ) | 0·53 | )estimated together | 9·3 |
| and/or 3:6- " " ) | | ) | |
| Monomethyl glucose | 0·29 | | trace. |
| Glucose | 0·11 | | trace. |

If the methylated 'whole' starch contains 20·5% of methylated amylose, it may be calculated from the

above data that the repeating-unit of the methylated amylopectin component consists of 20 glucose residues.

Hydrolysis of methylated elm starch. Fraction II (2·17 g.) was boiled gently on a water-bath with methanolic hydrogen chloride (100 ml.; 1%) for 7 hours. The solution was then cooled and cautiously neutralised with silver carbonate. The mixture was filtered and the insoluble residue of silver salts washed well with hot dry methanol. The filtrate and washings were then concentrated to a syrup which was dried in a vacuum over $P_2O_5$. Yield, 2·41 g. (96% of theoretical).

The syrupy glycosides were hydrolysed by heating at 95-100° with hydrochloric acid (100 ml.; 2%) for 14 hours. After cooling, the solution was neutralised with silver carbonate, filtered, and silver was then removed with hydrogen sulphide. The solution was filtered through a layer of "filter-cel" and concentrated to a syrup under reduced pressure at 40°. Yield, 2·18 g. (92·3% of theoretical yield from the methylated polysaccharide). The syrup partially crystallised on standing for 2-3 days.

Separation of the methylated glucoses on a cellulose column. A column of powdered cellulose (67 x 2·2 cm.) was prepared and washed as described by Hough, Jones and Wadman (233). The solvent selected for the

operation was a mixture of purified light petroleum (b.p. 100-120°) (50%) and n-butanol (50%), saturated with water. The mixture of methylated glucoses (2·18 g.) from the above hydrolysis was dissolved in this solvent (5 ml.) and the solution added dropwise to the centre of the top of the column, each drop being allowed to soak in before the next was added. When the addition of the solution was completed, the column was allowed to stand undisturbed for 1 hour and then a thin layer of powdered cellulose was tamped into position on the surface of the column followed by a thin layer of cotton wool. The 'constant-level' reservoir was filled with the solvent and inverted over the top of the column. The solvent issuing from the base of the column was collected in the receiving tubes which were changed at 12-minute intervals by means of the automatic device. A total of 300 tubes was collected, each tube containing 4-5 ml. of the eluate. The contents of every tenth tube was concentrated in a glass dish over a water-bath and any residue was dissolved in a little acetone and small portions of each solution transferred in chronological order to the starting-lines of paper chromatograms which were then irrigated in the usual manner. The residues in the glass dishes were returned quantitatively to the respective tubes. The chromatograms were developed with aniline oxalate

and the distribution of the sugars in the tubes thus determined.

Tubes 20-30 contained a sugar corresponding to 2:3:4:6-tetramethyl glucose and after a gap of 20 tubes, 2:3:6-trimethyl glucose appeared (50-90). A further gap of 10 tubes was followed by the appearance of an apparently homogeneous dimethyl glucose (100-130), then a mixture of dimethyl glucoses (130-160) followed by an apparently homogeneous dimethyl glucose (160-200). Tubes 200-300 contained no reducing sugars. The column was finally washed with water (500 ml.) to obtain traces of monomethyl glucose and glucose. The tubes were grouped appropriately and the solvent removed at 35°/20 mm. The residues in each case were dissolved in water and filtered through 'filter-cel.' The filtrates were treated with a little charcoal at 35° for 15 minutes and refiltered through 'filter-cel.' The solutions were evaporated at 40°/20 mm. and the residual syrups dissolved in acetone and filtered to remove traces of waxy impurities. After the removal of acetone, the syrups were dried in vacuo over $P_2O_5$ for several days. The following fractions were thus obtained: (1) tetramethyl glucose (0.1911 g.); (2) trimethyl glucose (1·8032 g.); (3) dimethyl glucose (0·0581 g.); (4) mixed dimethyl glucoses (0·0658 g.); (5) dimethyl glucose (0·0816 g.); (6) monomethyl glucose and

glucose (0·0195 g.).

Examination of the fractions. Fraction (1). The syrup showed no tendency to crystallise over a period of 2 weeks although examination on a paper chromatogram indicated the presence of one sugar only ($R_G$, 1·0) which corresponded to an authentic sample of 2:3:4:6 tetramethyl glucose. Quantitative estimation of the tetramethyl glucose by hypoiodite oxidation (232,240) indicated the presence of 0·0949 g. of that sugar in the syrup, the remainder of the syrup being represented mainly by trimethyl methylglucoside.

Hydrolysis of fraction (1). Fraction (1) (0·1857 g.) was heated at 95-100° with hydrochloric acid (17 ml.; 2%) for 10 hours. The products were isolated as described previously for the bulk hydrolysis of the methylated starch. Yield, 0·162 g. (90% of theoretical).

Separation of the products of hydrolysis of fraction (1) on a cellulose column. The apparatus, method and solvents used for the separation were the same as those previously described. The syrup (0·162 g.) was dissolved in the solvent (1·5 ml. of light petroleum (50%)-butanol (50%), saturated with water) and the solution transferred to the top of the well-washed cellulose column. The reservoir was filled with the solvent and the column developed by using the authmatic device for changing the receiver every

12 minutes. The eluate was collected in a total of 250 tubes each containing 4-5 ml., and the distribution of reducing sugars in these tubes determined as previously described. Tubes 20-40 were found to contain tetramethyl glucose. A gap of 20 tubes was followed by the appearance of trimethyl glucose (60-160). Although the remaining tubes (160-250) appeared to contain no reducing sugars, their contents were combined and evaporated to dryness. The residue examined on a paper chromatogram, developed with aniline oxalate gave two spots of roughly equal intensities corresponding with 2:3- and 3:6-dimethyl glucoses. The column was washed with water (500 ml.) and the eluate evaporated to dryness. No reducing sugars were detected when the residue was examined on a paper chromatogram.

The following fractions were isolated and dried as described previously: (1a), tetramethyl glucose (0·0885 g.); (1b) trimethyl glucose (0·0682 g.); (1c) dimethyl glucose (0·0051 g.).

Examination of fractions (1a) and (1b). Fraction (1a). The syrup crystallised almost completely after the removal of traces of solvent. Chromatographic examination indicated the presence of a single substance ($R_G$, 1·0) which corresponded to 2:3:4:6-tetramethyl glucose. Hypoiodite oxidation (232,240) indicated that the fraction was 97% pure. The crystals

(0·0847 g.) were recrystallised twice from light petroleum (b.p. 40-60°) and recovered in the form of long needles. Yield, 0·041 g.; m.p. 85-7°, not depressed on admixture with an authentic sample of 2:3:4:6 tetramethyl glucose; OMe, 51·9% (Calc. for $C_{10}H_{20}O_6$, 52·5%). $[\alpha]_D^{17^\circ}$ + 96° (initial) falling to $[\alpha]_D^{17^\circ}$ + 83° at equilibrium (c, 0·2 in water).

The quantity of 2:3:4:6 tetramethyl glucose recovered from the hydrolysate of the methylated starch was 91·7 (± 3·2) mg.

Fraction (1b). The syrup partially crystallised on drying. Paper chromatography indicated the presence of one reducing sugar only corresponding to 2:3:6 trimethyl glucose. Hypoiodite oxidation indicated that the fraction was 94·8% pure; OMe 39·2% (Calc. for $C_9H_{18}O_6$, 41·9%); $[\alpha]_D^{18^\circ}$ + 85° (initial) falling to $[\alpha]_D^{18^\circ}$ + 67° at equilibrium (c, 0·5 in water).

Rotation of fraction (1b) in cold methanolic hydrogen chloride. Fraction (1b)(0·031 g.) was dissolved in methanolic hydrogen chloride (10 ml.; 1%) at room temperature. The rotation of the solution in a 2 dm. polarimeter tube was observed at intervals during 8 hours after which no further change occurred. The following values were recorded:- $[\alpha]_D^{17^\circ}$ + 64° (initial) to $[\alpha]_D^{17^\circ}$ - 36° (final, 8 hours).

Fraction (2) partially crystallised on drying. Examination on a paper chromatogram indicated the

presence of a single sugar corresponding to 2:3:6-trimethyl glucose. Hypoiodite oxidation indicated that the fraction was 93·5% pure. The crude material (53·5 mg.) was treated with hydrochloric acid (3 ml.; 2%) at 95-100° for 8 hours. The solution was neutralised and evaporated to a syrup as previously described. Examination of the residue on a paper chromatogram revealed very faint traces of dimethyl glucoses along with an intense spot corresponding to 2:3:6-trimethyl glucose. Fraction (2) (1·6 g.) was recrystallised twice from dry ether and the sugar recovered in the form of fine needles. Yield, 0·71 g.; m.p. 113-115° with previous softening, alone or on admixture with an authentic specimen of 2:3:6-trimethyl-D-glucopyranose; OMe, 41·2% (Calc. for $C_9H_{18}O_6$, 41·9%); $[\alpha]_D^{19°}$ + 98° (initial) falling to $[\alpha]_D^{19°}$ + 68° at equilibrium (c, 1·0 in water).

Rotation of recrystallised fraction (2) in cold methanolic hydrogen chloride. The crystals (0·11 g.) were dissolved in methanolic hydrogen chloride (10 ml.; 1%) at room temperature. The rotation was observed at intervals during 14 hours after which no further change occurred. The following values were recorded: $[\alpha]_D^{17°}$ + 69° (initial) to $[\alpha]_D^{17°}$ - 42° (final, 14 hours).

Fraction (3) did not crystallise on standing. Hypoiodite oxidation indicated that the syrup contained 95·6% of dimethyl glucose. The position and colour

of the spot on a paper chromatogram developed with aniline oxalate corresponded to 2:3-dimethyl glucose.

Fraction (4) did not crystallise on standing. Hypoiodite oxidation indicated that the syrup contained 94·8% of dimethyl glucose. Examination by paper chromatography indicated that the syrup contained at least two dimethyl glucoses. These two components, separated on a quantitative paper chromatogram by irrigation with butanol-ethanol-water mixture for 45 hours, were estimated by the method of Hirst, Hough and Jones (232). The results indicated that fraction (4) consisted of 53% of 2:3-dimethyl glucose. The remainder was possibly represented by 3:6- and/or 2:6-dimethyl glucoses.

Fraction (5) did not crystallise on standing. Hypoiodite oxidation indicated that the syrup contained 94·3% of dimethyl glucose. Paper chromatography indicated the presence of one sugar only, but it is possible that the syrup consisted of 3:6- and/or 2:6-dimethyl glucoses.

Further investigations of fractions (3), (4) and (5) are in progress.

Fraction (6) was examined on a paper chromatogram. Development with aniline oxalate revealed the presence of glucose and another sugar ($R_G$, 0·20) which corresponded to a monomethyl glucose and which gave a red-brown spot. 3-Methyl glucose ($R_G$, 0·19) which gave a brown spot was used as the 'control' sugar.

The total quantities of the various sugars recovered from the column were as follows:-

| | |
|---|---|
| 2:3:4:6-tetramethyl glucose, | 0·0917 (± 0·0032)g. |
| 2:3:6-trimethyl glucose, | 1·7534 g. |
| 2:3-dimethyl glucose (?), | 0·0886 g. |
| 3:6 and/or 2:6-dimethyl glucose(?) | 0·1062 g. |
| methyl glucose + glucose | 0·0195 g. |
| Total recovery = | 2·0594 g. (i.e. |

94·5% of the possible recovery).

The limiting values for the "number of glucose residues per non-reducing end-group" in methylated elm amylopectin (viz. 20 ± 1) were obtained by calculations based upon the upper and lower limiting values for the recovery of tetramethyl glucose together with the total actual and the total possible recoveries of the methylated sugars from the column, respectively. The calculations were made upon the assumption that 20·5% of methylated amylose was present in the methylated 'whole' starch and that the methylated amylose yielded 0·4% of tetramethyl glucose upon hydrolysis. Due allowance was also made for the effects of demethylation of tetramethyl glucose by the reagents used for hydrolysis of the polysaccharide. The latter calculation was based upon the results of the following experiments.

The Partial Demethylation of 2:3:4:6-Tetramethyl- and 2:3:6-Trimethyl glucose. Samples of the appropriate sugars (50 mg.) were treated first with methanolic hydrogen chloride (1·5 ml.; 1%) in a sealed tube at 100° for 7 hours and then with aqueous hydrochloric acid (4 ml.; 2%) at 100° for 14 hours. The samples were thus subjected to slightly more drastic treatment than the methylated starch during hydrolysis. The products were isolated in the usual way (232) and the components of each syrup separated on paper chromatograms and estimated with buffered hypoiodite (232,240). The results are collected in the following table.

| Sample. | 0·01N. $Na_2S_2O_3$ (ml.) | Methylated sugar, %. |
|---|---|---|
| 2:3:4:6-tetramethyl glucose | | |
| tetramethyl glucose. | 2·248 | 98·3 |
| trimethyl glucose. | 0·041 | 1·7 |
| 2:3:6-trimethyl glucose | | |
| trimethyl glucose. | 2·611 | 98·5 |
| dimethyl glucose. | 0·042 | 1·5 |

TABLE X.

These figures suggest that the amount of demethylation suffered by the products of hydrolysis of methylated starch under the above conditions was slight. Hence, the yield of "end-group," compensated for the effects of demethylation, did not differ materially from the observed yield. The data in Table X also

indicates that the greater part of the relatively high yield of dimethyl glucoses (total, 9·5%) did not arise by demethylation during hydrolysis. The yield of dimethyl glucoses in excess of that which can be accounted for directly on the basis of the Haworth-Hirst formula for amylopectin probably arose, therefore, by undermethylation of the starch.

## S U M M A R Y.

1. The difficulties encountered in preliminary investigations on wood starches are described. The presence of gallotannin, apparently strongly adsorbed on the granules, was found to be responsible for serious interference particularly with the results of potentiometric iodine titration for the determination of amylose and with the results of periodate oxidation for the determination of the number of glucose residues in the starches per non-reducing end-group. The reactivity of gallotannin with iodine has been shown to be responsible for erroneous values of molecular weight determination of the starch samples by means of alkaline hypoiodite oxidation.

2. A technique for overcoming the interference of the impurities with potentiometric iodine titrations was developed and the amylose contents of the wood starches were shown to vary between 14% and 20%

3. Attempts to remove the impurities from the wood starch samples are described. Solvent extraction of the granules with boiling 85% aqueous methanol was found to be the most effective method of removing the reactive gallotannin without simultaneous degradation of the polysaccharides.

Periodate titration of gallotannin-free elm

sapwood starch revealed that the repeating-unit of the amylopectin component consisted of some 20 glucose residues after compensation had been made for the comparatively slight reactivity of lignin which was thought not to have been removed by the process of solvent extraction.

4. Samples of amylose were isolated by fractionation of elm and oak sapwood starches by slow cooling of hot dispersions of the starches in 15% aqueous pyridine. The crude amyloses obtained from the initial fractionations were each reprecipitated three times from butanol-saturated water. The amylose-butanol complexes were isolated in typical spherocrystalline form. The recovered amyloses were shown to adsorb iodine to the extent of 19-20% of their own weight.

5. Acetylation of wood starches yielded products the general properties of which were characteristic of other similarly acetylated starches.

6. Methylation of elm sapwood starch yielded a product which closely resembled other similarly methylated starches. The material possessed relatively high specific viscosity in m-cresol indicating a molecular weight of the order of $5 \times 10^5$. Hydrolysis of methylated elm starch and chromatographic separation

of the products on a cellulose column yielded a quantity of 2:3:4:6-tetramethyl glucose consistent with a repeating-unit chain-length for the amylopectin component of 20 $\pm$ 1 glucose residues on the basis of the Haworth-Hirst 'laminated' formula for amylopectin.

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